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*Wellen, 5/31/01*

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List of personnel supported on this grant

Three published papers that were supported on this application

1. Juhl et al 1997 J Biol Chem
2. Hsieh et al 2000 Int J of Cancer
3. Aigner et al 2001 Oncogene



## **INTRODUCTION**

Breast cancer tissue consists of a mixture of autonomously proliferating tumor cells and supportive normal tissue recruited by them. Generally speaking, the primary carcinogenic events lead to uncontrolled growth of transformed cells. The secondary development into breast cancer then induces and requires a network of growth signals between the tumor cells and the normal surrounding host tissue (1,2). These growth signals are primarily processed at the cell membrane by specific receptors and activation of the receptor triggers a cascade of downstream events. Aberrations in this signal transduction cascade can lead to deregulated cellular proliferation, differentiation, and ultimately to tumorigenesis. The most frequently implicated receptors and growth factors in human cancers are members of the EGF receptor family and their ligands (3). In addition to the EGF receptor (HER-1), this family includes HER-2 (neu/erbB-2), HER-3 (erbB-3), and the more recently discovered HER-4 (erbB-4) receptor (4-6). These receptors share a common molecular architecture and are able to form heterodimers with each other (7). Heterodimeric formation is probably the mechanism that allows ligands to increase tyrosine phosphorylation of the different receptors of the HER family (8). So far known ligands include EGF, TGF- $\alpha$ , cripto-1, amphiregulin (all binding to EGFR) and heregulin (NDF) which binds to HER-3 and HER-4. Until now no HER-2 specific ligand has been found, although there are at least three distinct mechanisms to activate the transforming potential of HER-2. This includes a point mutation within the transmembrane domain, truncations of catalytic sequences at both the cytoplasmic and the extracellular domains, or overexpression of the structurally normal gene (8).

HER-2 gene amplification and receptor overexpression has been detected in several human adenocarcinomas (including breast, ovarian, lung and gastric) at a frequency between 20-40% (9-11). The role of HER-2 as prognostic marker in breast cancer has been the subject of numerous clinical studies. So far the results from these studies vary considerably, but there is a general consensus indicating a correlation between HER-2 overexpression, axillary lymph node involvement and worse patient survival (11-14). The poor patient outcome in HER-2 positive patients is associated with a correlation between HER-2 expression and development of resistance to chemotherapy with alkylating agents like cisplatin and doxorubicin (14-18). On the other hand data from the CALGB 8869 study (19) indicate an altered dose response relationship of doxorubicin in HER-2 overexpressing patients. These patients benefited dramatically by increasing doses of doxorubicin, whereas increasing doses of doxorubicin had no effect in patients expressing low levels of HER-2. Finally, Slamon et al have recently shown that in clinical trials anti-HER-2 antibody therapy is beneficial for breast cancer patient survival and disease recurrence (20,21).

### **Overview of the goals pursued under this award:**

Our studies were aimed at elucidating the contribution of HER-2 to breast cancer growth and progression to hormone independence as well as the development of resistance to treatment with cytotoxic drugs, anti-hormones and anti-HER-2 antibodies. Ultimately we wish to develop this understanding into novel therapeutic strategies. As a major tool we have used gene expression of a HER-2 truncated form and gene-specific targeting of HER-2 with hammerhead-ribozyme expression constructs, a technology which we have applied successfully in the targeting of the growth factor pleiotrophin (22) and a number of other genes (23-26).

## **BODY**

**Work accomplished during the most recent award cycle:**

### **GOAL 3: The role of HER-2 overexpression in the development of resistance to treatment with cytotoxic drugs**

#### **Background:**

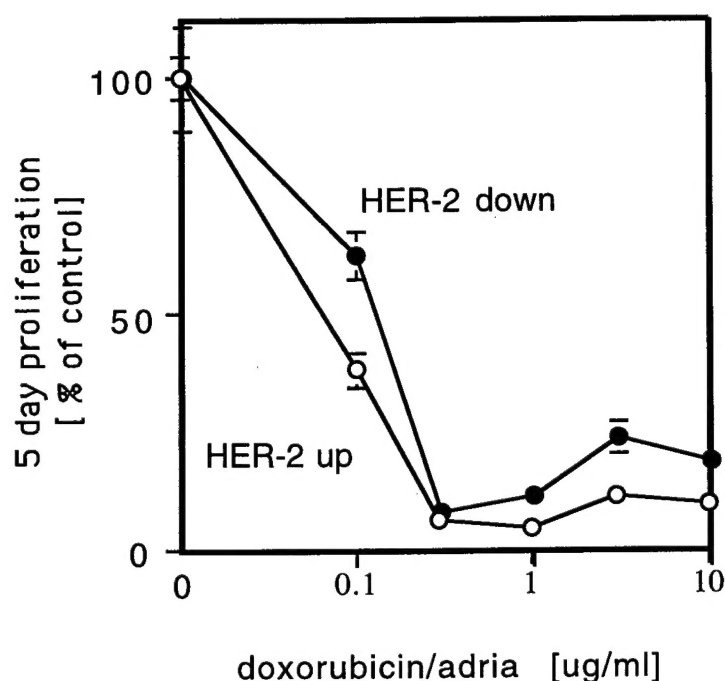
Hormones and growth factors define the capacity of human breast cancer to grow and metastasize. One of the essential requirements for the development of breast cancer are circulating steroid hormones and one of the most widely used drug therapies of breast cancer with the anti-estrogen tamoxifen is based on this fact. Furthermore, growth factor gene expression can supplement for hormone stimulation and thus contribute to hormone-independent cancer growth as well as to resistance to anti-hormone therapy or to cytotoxic drugs (reviewed e.g. in (27)).

For this experimental series we used the MCF-7/tetHER-2ribozyme cells that were described in previous reports. In these cells we can regulate HER-2 levels by transfected ribozymes under tetracycline regulation. The endogenous HER-2 levels can be modified to be normal ("up") in the presence of tetracycline (ribozyme off) or reduced by 90% ("down") in the absence of tetracycline. We then included the topoisomerase II inhibitor adriamycin (also named doxorubicin) since this is a mainstay therapy in breast cancer and sensitivity of tumors to this drug have been suggested to be related to expression of HER-2 (see Ref. 19). We used proliferation as a measure of the efficacy of the drug.

Fig. 1 (see next page) shows the data from one of an experimental series. We found a significant ( $p < 0.01$ ) decrease in the sensitivity of the cells to doxorubicin when HER-2 was downmodulated. A concentration of the cytotoxic drug that inhibits cells under control conditions (= "HER-2 up") by 65% only inhibits by 35% when HER-2 is downregulated and an approximately 3-fold rightshift is observed. This very clearly shows that the cytotoxic effects of doxorubicin are blunted when HER-2 is downmodulated. Interestingly, this was NOT observed with other cytotoxic agents (reported in our first report).

#### **CONCLUSION:**

These experiments were done in isogenic cells in which we only modulate the endogenous HER-2 levels by virtue of an added regulator of a transfected ribozyme (the tetracycline system). We thus conclude that the difference in topoisomerase II inhibitor sensitivity is due to changes in HER-2 function.



**Fig. 1. Effect of HER-2 reduction by tetracycline-regulated ribozymes on MCF-7 cell sensitivity to adriamycin (=doxorubicin)**

MCF-7/tetHER-2ribozyme cells as described in previous reports were used for the experiments. Proliferation was measured after 5 days. The cells were grown in the absence or presence of tetracycline to generate the "HER-2 down" or "HER-2 up" phenotype respectively as described. In addition they were grown without and with different concentrations of doxorubicin (=adriamycin = adria) as indicated in micrograms per ml (= ug/ml).

10% charcoal-stripped fetal calf serum was included in the growth media. ANOVA analysis was performed to analyze differences between the groups and the results are given in the text. Mean  $\pm$  SEM values are shown for each data point which was run in sixtuplicate.

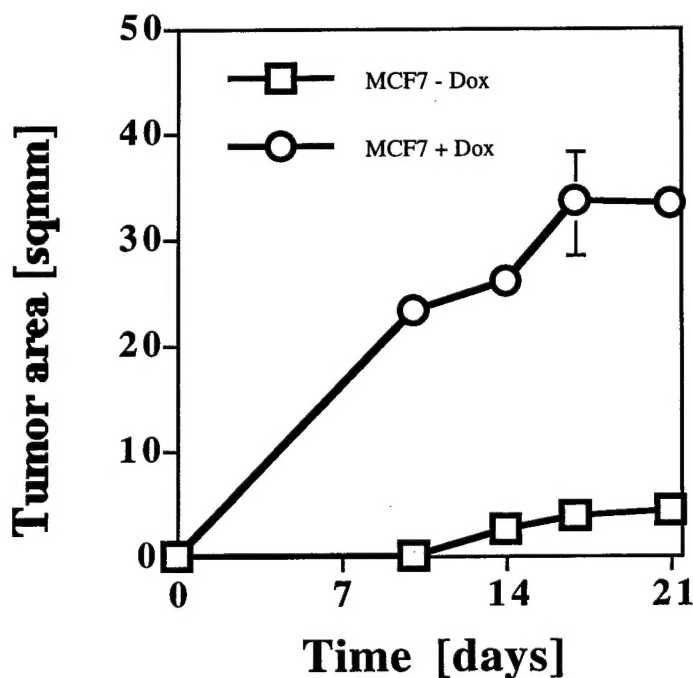
## GOAL 4: effect of HER-2 downregulation in tumors *in vivo*

### Dependence of estrogen-dependent tumor growth in animals on HER-2

#### Background:

As described in the last report, we tested whether the dependence of estrogen-induced colony formation of MCF-7 cells on the presence of HER-2 was affected by steroid hormones present in fetal-calf **serum**. The rationale for these experiments was that serum contains a number of (unknown) factors that may affect the sensitivity to hormones and growth factors and we thus repeated the experiments with charcoal treated ("stripped") **serum** from which steroid hormones have been removed. Upon downmodulation of the HER-2 by removal of tetracycline and thus turning the HER-2-targeted ribozyme on, the estrogen lost its efficacy and estrogen stimulation was reduced significantly. From those *in vitro* studies we concluded that the growth effects of estrogen in MCF-7 cells depend on an intact HER-2 pathway.

We tested next whether this would translate into a difference in tumor growth of these cells *in vivo*. The data are shown in Fig.2.

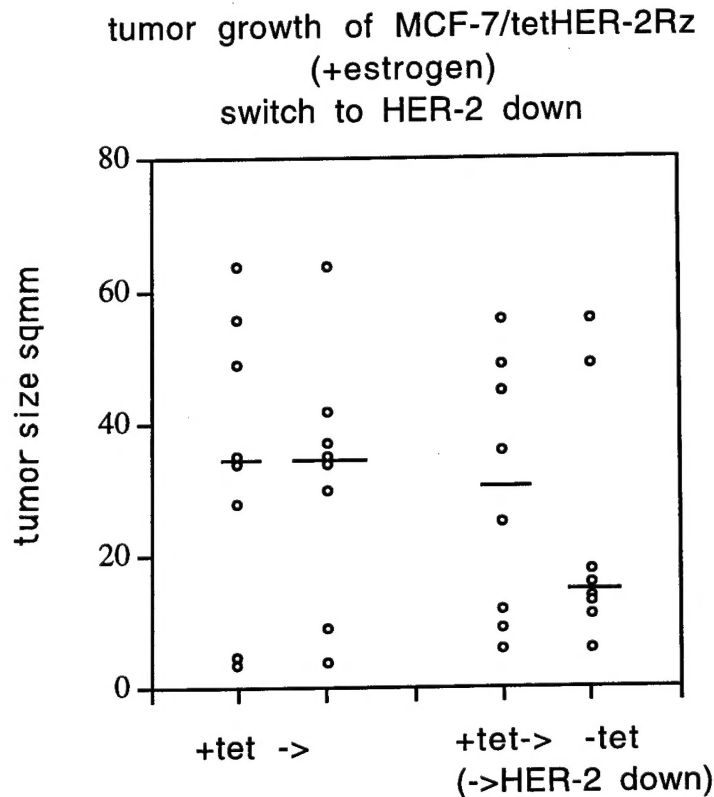


**Fig. 2. Effect of HER-2 reduction by tetracycline-regulated ribozymes on estradiol-dependent tumor growth of MCF-7 cells.**

MCF-7/tetHER-2ribozyme cells as described in previous reports were used for the experiments. Animals with estradiol pellets ( $n = 8$  +dox and  $n = 4$  -dox) were inoculated with 10 million tumor cells in 2 sites per animal and either fed a doxycycline containing diet to keep the ribozyme off or a control diet. Tumor size was monitored using calipers and mean  $\pm$  SEM values are shown for each data point (error bars smaller than the symbol size do not show up). ANOVA showed a significant difference between the two groups ( $p < 0.01$ )

As shown in Figure 2 there is a striking (and statistically significant) difference in tumor growth amongst the two groups. This rules out that circulating or stromal growth factors in the animals could compensate for the loss of HER-2 signaling.

As an extension of the above setting, we also used this experimental paradigm to test whether the turning off of HER-2 in established tumors would reduce further tumor expansion.



**Fig. 3. Effect of HER-2 reduction in established tumors by tetracycline-regulated ribozymes.**

MCF-7/tetHER-2ribozyme cells as described above were used for the experiment. Animals with estradiol pellets ( $n=8$  + tet) were inoculated with 10 million tumor cells in 2 sites per animal and fed a doxycycline-containing diet (doxycycline is a more stable tetracycline) to keep the ribozyme off. Upon tumor establishment they were randomized to continue on the tetracycline (+ tet ->) or switched to non-tetracycline containing diets (+tet -> -tet). Tumor size of individual animals is shown as is the median tumor size. We observed a down-ward trend when HER-2 was downregulated.

**Conclusion:** From the above data we conclude that HER-2 is the rate-limiting modulator for estrogen-induced tumor growth. Circulating or stromal growth factors can not complement the loss of this major signaling molecule.

## **KEY RESEARCH ACCOMPLISHMENTS**

Our studies were aimed at elucidating the contribution of HER-2 to breast cancer growth and progression to hormone independence as well as the development of resistance to treatment with cytotoxic drugs, anti-hormones and anti-HER-2 antibodies. Ultimately we wish to develop this understanding into novel therapeutic strategies. As a major tool we have used gene expression of a HER-2 truncated form and gene-specific targeting of HER-2 with hammerhead-ribozyme expression constructs, a technology which we have applied successfully in the targeting of the growth factor pleiotrophin (22) and a number of other genes (23-26).

We have studied

under **goal 1**: whether downregulation of endogenous HER-2 expression by targeting with HER-2 ribozymes will affect the *in vitro* growth and HER-2 signal transduction pathways of HER-2 overexpressing cancer cells

**We found that HER-2 is rate limiting for growth factor induced growth that relies on ligands for the HER-1 / -3/ -4 family (EGF, heregulin.**

**The major pathway affected is apoptosis.**

under **goal 2**: to what extent downregulation of HER-2 expression affects the sensitivity of breast cancer cells to treatment with steroid hormones

**This was the most surprising finding: Estrogen needs HER-2 for its activity in vitro and for tumor growth in animals. Furthermore, the pathway affected is not along the cell cycle but the rescue from apoptosis.**

under **goal 3**: the role of HER-2 overexpression in the development of resistance to treatment with cytotoxic drugs

**We found that in particular doxorubicin sensitivity is dependent on the presence of intact HER-2. Downregulation of HER-2 increases the resistance to doxorubicin approximately 3-fold.**

under **goal 4**: whether downregulation in tumors *in vivo* of endogenous HER-2 by targeting with tetracycline regulated HER-2 ribozyme constructs will affect tumor growth in animals

**HER-2 overexpression is rate-limiting for overall tumor growth; also, that (surprisingly) for estrogen-induced tumor growth HER-2 expression is required.**

under **goal 5**: whether expression of a truncated HER-2/ECD is involved in the development of resistance to therapy with HER-2 antibodies such as muAb4D5.

**Surprisingly, expression of a truncated HER-2 splice variant acts as an endogenous inhibitor of tumor cell growth induced by growth factors and serum and thus would act in conjunction with a HER-2-targeted therapy.**

## **REPORTABLE OUTCOMES**

- Estrogen action in breast cancer appears to need intact HER-2 (a paper containing these data is in preparation for submission; List et al. )
- HER-2 expression is rate-limiting for tumor growth (published in 1997 in J. Biol Chem; attached as Juhl et al.)
- Growth factor signaling through the HER-family is strictly dependent on the presence of HER-2 (published in 2000 in the Int J of Cancer; attached as Hsieh et al.)
- A truncated version of HER-2 acts to inhibit tumor cell growth (published in 2001 in Oncogene; attached as Aigner et al.)

## **CONCLUSIONS**

We analyzed the role of HER-2 for the growth of human breast cancer cells and found surprisingly that estrogen needs HER-2 presence for its action. Furthermore, we were able to demonstrate that sensitivity to doxorubicin is dependent on intact HER-2 and we found that the truncated HER-2 reported by others in fact is an inhibitor of the HER-family pathway.

## REFERENCES

1. Cross, M., and Dexter, T. M. (1991) *Cell* **64**, 271-280
2. Liotta, L. A., Steeg, P. S., and Stetler-Stevenson, W. G. (1991) *Cell* **64**, 327-336
3. Ullrich, A., and Schlessinger, J. (1990) *Cell* **61**, 203-212
4. Kraus, M. H., Issing, W., Miki, T., Popescu, N. C., and Aaronson, S. A. (1989) *Proc Natl Acad Sci USA* **86**, 9193-9197
5. Plowman, G. D., Culouscou, J. M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G., and Shoyab, M. (1993) *Proc Natl Acad Sci USA* **90**, 1746-1750
6. Kraus, M. H., Fedi, P., Starks, V., Muraro, R., and Aaronson, S. A. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2900-2904
7. Carraway III, K. L., and Cantley, L. C. (1994) *Cell* **78**, 5-8
8. Bacus, S. S., Zelnick, C. R., Plowman, G., and Yarden, Y. (1994) *Am J Clin. Pathol.* **102**, S13-S24
9. Yokata, J., Yamamoto, T., Toyoshima, K., Teroda, M., Sugimura, T., Battifora, H., and Cline, M. J. (1986) *Lancet* **5**, 765-766
10. Lemoine, N. R., Staddon, S., Dickson, C., Barnes, D. M., and Gullick, W. J. (1990) *Oncogene* **5**, 237-239
11. van de Vijver, M., van de Berselaar, R., Devilere, P., Cornelesse, C., Peterse, J., and Nusse, R. (1987) *Mol Cell Biol* **7**, 2019-2023
12. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W., Ullrich, A., and McGuire, R. L. (1987) *Science* **235**, 177-182
13. Varley, J. M., Swallow, J. E., Brammar, W. J., Whittaker, J. L., and Walker, R. A. (1987) *Oncogene* **1**, 423-430
14. Zhou, D., Battifora, H., Yokota, J., Yamamoto, T., and Cline, M. J. (1987) *Cancer Res* **47**, 6123-6125
15. Allred, D. C., Clark, G. M., Tandon, A. K., Molina, R., Tormey, D. C., Osborne, C. K., Gilchrist, K. W., Mansour, E. G., Abeloff, M., and Eudey, L. (1992) *Journal of Clinical Oncology* **10**, 599-605
16. Smith, K., Houlbrook, S., Greenall, M., Carmichael, J., and Harris, A. L. (1993) *Oncogene* **8**, 933-938
17. Tsai, C. M., Chang, K. T., Perng, R. P., Mitsudomi, T., Chen, M. H., Kadoyama, C., and Gazdar, A. F. (1993) *J Natl. Cancer Inst.* **85**, 897-901
18. Tsai, C. M., Yu, D., Chang, K. T., Wu, L. H., Perng, R. P., Ibrahim, N. K., and Hung, M. C. (1995) *J Natl. Cancer Inst.* **87**, 682-684
19. Muss, H. B., Thor, A. D., Berry, D. A., Kute, T., Liu, E. T., Koerner, F., Cirrincione, C. T., Budman, D. R., Wood, W. C., Barcos, M., and Henderson, I. C. (1994) *N. Engl. J Med.* **330**, 1260-1266
20. Pegram, M. D., and Slamon, D. J. (1999) *Semin Oncol* **26**(4 Suppl 12), 89-95.
21. Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., and Norton, L. (2001) *N Engl J Med* **344**(11), 783-92.
22. Czubayko, F., Riegel, A. T., and Wellstein, A. (1994) *J. Biol. Chem.* **269**, 21358-21363
23. Czubayko, F., Schulte, A. M., and Wellstein, A. (1998) in *Therapeutic Applications of Ribozymes* (Scanlon, K. J., ed), pp. 311-328, Humana Press, Totowa N.J.



24. Schulte, A. M., Lai, S., Kurtz, A., Czubayko, F., Riegel, A. T., and Wellstein, A. (1996) *Proc.Natl.Acad.Sci.USA* **93**, 14759-14764
25. Czubayko, F., Downing, S., Hsieh, S. S., Goldstein, D., Lu, P., Trapnell, B., and Wellstein, A. (1997) *Gene Therapy* **4**, 943-949
26. Czubayko, F., Liaudet-Coopman, E. D. E., Aigner, A., Tuveson, A. T., Berchem, G., and Wellstein, A. (1997) *Nature Medicine* **3**, 1137-1140
27. Dickson, R., and Lippman, M. E. (1987) *Endocr.Rev.* **8**, 29-43
28. Sumantran, V. N., Ealovega, M. W., Nunez, G., Clarke, M. F., and Wicha, M. S. (1995) *Cancer Res* **55**, 2507-2510
29. Juhl, H., Downing, S. G., Wellstein, A., and Czubayko, F. (1997) *J Biol Chem* **272**, 29482-29486

## **APPENDICES**

### **Personnel involved in the studies:**

Frank Czubayko, MD, Professor of Pharmacology

Hartmut Juhl, MD PhD associate Professor of Oncology

Susie Hsieh, PhD, Research Scientist

Heinz-Joachim List, PhD, Research Instructor

Anton Wellstein, MD PhD, Professor of Oncology and Pharmacology

### **Papers attached and published under this award**

Juhl, H. et al (1997) J Biol Chem

Hsieh et al (2000) Int J of Cancer

Aigner et al (2001) Oncogene

### **Papers in preparation under this award (not completed yet)**

List et al (2001) to be submitted to Proc Natl Acad Sci

## ERBB-2 EXPRESSION IS RATE-LIMITING FOR EPIDERMAL GROWTH FACTOR-MEDIATED STIMULATION OF OVARIAN CANCER CELL PROLIFERATION

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Over-expression of the *ErbB-2* proto-oncogene frequently coincides with an aggressive clinical course of certain human adenocarcinomas. The *ErbB-2* receptor is a member of the *ErbB* family of growth factor receptors, and within this complex signaling network, *ErbB-2*-containing heterodimers are preferentially formed. To assess whether *ErbB-2* is a critical component in epidermal growth factor (EGF)-mediated stimulation of tumor cell proliferation, we used as a model SK-OV-3 ovarian cancer cells, which over-express EGF receptor (EGFR) and *ErbB-2* receptors. In these cells, we reduced *ErbB-2* mRNA and protein expression by transfection with *ErbB-2*-targeted hammerhead ribozymes and generated cell lines expressing different levels of *ErbB-2*. In SK-OV-3 cells, *ErbB-2* expression conferred a growth advantage and soft agar experiments revealed that *ErbB-2* was rate-limiting for anchorage-independent growth. The induction of colony formation by EGF was completely abrogated in *ErbB-2*-depleted cells, despite unchanged expression levels and tyrosine phosphorylation of the EGFR. The duration of EGF-mediated c-Fos mRNA up-regulation was decreased in parallel with loss of *ErbB-2* expression. Furthermore, the rate of spontaneous apoptosis was increased in *ErbB-2*-depleted cells. Our results demonstrate that in human ovarian cancer cells the EGFR-*ErbB-2* heterodimer, and not the EGFR homodimer, can be rate-limiting for EGF-mediated proliferation, thus suggesting that the oncogenic activity of *ErbB-2* in human tumors is due in part to its ability to increase the growth response to stroma-derived EGF-like growth factors. *Int. J. Cancer* 86:644–651, 2000.  
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Receptor tyrosine kinases (RTKs) are major mediators of growth and differentiation signals. The *ErbB* family of RTKs consists of 4 receptors: *ErbB-1* [also called epidermal growth factor receptor (EGFR)], *ErbB-2* (also called HER-2/neu), *ErbB-3* and *ErbB-4* (HER-3 and HER-4, respectively) (Alroy and Yarden, 1997). The *ErbB* receptor family establishes an extensive signaling network that controls diverse biological processes such as embryogenesis and oncogenesis (Alroy and Yarden, 1997). Members of the family have been implicated in malignant transformation and are over-expressed in a variety of human tumors. EGFR over-expression has been documented in human breast carcinomas, glioblastomas and epidermoid carcinomas (Salomon *et al.*, 1995). *ErbB-2* over-expression has been reported in 20% to 30% of human adenocarcinomas of the breast, ovary, endometrium, lung and stomach and linked to an unfavorable prognosis in patients with breast and ovarian cancer (Slamon *et al.*, 1989). Analysis of *ErbB-3* and *ErbB-4* expression in human cancer is at an early stage but appears to be more limited (Carraway and Cantley, 1994).

Simultaneous over-expression of at least 2 *ErbB* receptors has been frequently observed in human cancer (Salomon *et al.*, 1995) and is currently believed to be a major cause of the oncogenic activity of *ErbB* receptors. *ErbB* receptors are activated by a large group of ligands (Salomon *et al.*, 1995) that can be divided into 2 groups based on their binding specificities. EGF-like ligands bind primarily to the EGFR and include EGF, transforming growth factor- $\alpha$ , amphiregulin, betacellulin, heparin-binding EGF and epiregulin. The second group consists of the heregulin growth factor family [also called neu differentiation factors (NDFs) or neuregulins] and binds to *ErbB-3* and *ErbB-4* (Carraway and Cantley, 1994; Tzahar *et al.*, 1994). Ligand-induced receptor dimerization adds another layer of complexity to the system; *e.g.*, *ErbB-3* is a

low-affinity receptor for heregulin (Tzahar *et al.*, 1994), but heterodimerization with *ErbB-2* leads to a conformational change and a much higher heregulin-binding affinity (Tzahar *et al.*, 1994). Furthermore, the heterodimeric *ErbB-2*-*ErbB-3* complex can bind EGF-like ligands (Alimandi *et al.*, 1997), which were previously believed to bind exclusively to the EGFR. The complexity of ligand-receptor interactions is further increased by the finding that heregulin simultaneously binds to *ErbB-2* and *ErbB-3* (Tzahar *et al.*, 1997) and that this ligand bivalence may enable further signal diversification through selective recruitment of different receptor combinations. Within the *ErbB* signaling network, *ErbB-2* has emerged as the preferred low-affinity subunit of all other *ErbB* receptors (Graus-Porta *et al.*, 1997), and heterodimeric receptor combinations containing *ErbB-2* show superior signal-transducing and cell growth-stimulating capabilities (Graus-Porta *et al.*, 1995; Karunakaran *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996). There is strong evidence that *ErbB-2* over-expression confers a growth advantage to tumor cells in an autocrine fashion (Alroy and Yarden, 1997; Salomon *et al.*, 1995). In addition, it has been speculated that *ErbB-2* is responsible for the increased responsiveness of tumor cells to stroma-derived EGF-like growth factors (Lewis *et al.*, 1996), a mechanism that may explain the correlation between *ErbB-2* over-expression, increased sensitivity to radio- and chemotherapy and enhanced metastatic potential. A recombinant human anti-*ErbB-2* monoclonal antibody (Mab) (Herceptin; Genentech, San Francisco, CA) has been developed as a novel therapeutic agent and is currently under investigation in various clinical trials (Shak, 1999). So far, results in metastatic breast cancer show an overall response rate of 14%, with the most beneficial effect in patients with the highest extent of *ErbB-2* protein over-expression.

To study this complex *ErbB*-signaling network, models have been devised that rely either on over-expression of *ErbB* receptors in cells with a receptor-negative background (Pierce *et al.*, 1988; Pinkas-Kramarski *et al.*, 1996; Alimandi *et al.*, 1997) or on the targeted inactivation of endogenously over-expressed *ErbB* receptors. Toward the second approach, various strategies have been developed, including *ErbB-2* anti-sense targeting (Vaughn *et al.*, 1995), inhibitory antibodies (Hurwitz *et al.*, 1995) and intracellular expression of *ErbB-2*-directed single-chain antibodies (Graus-Porta *et al.*, 1995).

We have shown that hammerhead ribozymes can down-regulate *ErbB-2* in over-expressing tumor cells (Juhl *et al.*, 1997; Czubayko *et al.*, 1997). Here, we use that approach to abrogate *ErbB-2*

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expression in human SK-OV-3 ovarian cancer cells that over-express only EGFR and ErbB-2 receptors and in turn can be stimulated by EGF but not by heregulin-like growth factors. We show that the ErbB-2-containing heterodimer, and not the EGFR homodimer, can be rate-limiting for EGF-mediated stimulation of ovarian cancer cell proliferation.

#### MATERIAL AND METHODS

##### Plasmids and generation of constructs

The ErbB-2-targeted hammerhead ribozymes RzA, RzB and RzC were designed and cloned into the pRc/CMV expression plasmid (Invitrogen, San Diego, CA) as described (Juhl *et al.*, 1997; Czabayko *et al.*, 1997). In brief, the following ribozyme-coding sense and anti-sense oligonucleotides were annealed together and ligated into the HindIII-restriction site of pRc/CMV: RzA, 5'-agcttGCCCCTGTCTGATGAGTCCGTTAGGACGAA-ACAGGGGTGa-3' (sense) and 5'-agcttCACCCCTGTTTCG-TCCTAACGGACTCATCAGACAGGGGCA-3' (anti-sense); RzB, 5'-agcttCAAGACCACCTGATGAGTCCGTTAGGACGAA-ACCAGCAGa-3' (sense) and 5'-agcttCTGCTGGTTCGTCCTA-ACGGACTCATCAGTGGTCTTGa-3' (anti-sense); and RzC, 5'-agcttGGGACTCTTCTGATGAGTCCGTTAGGACGAAACC-AGCAGCa-3' (sense) and 5'-agcttCGTGCTGGTTCGTCCTA-ACGGACTCATCAGAAAGAGTCCCa-3' (anti-sense) (with lowercase letters indicating HindIII-restriction site overhangs, boldface uppercase letters showing ErbB-2-specific anti-sense regions and underlined upper-case letters indicating the hammerhead ribozyme core sequence). The resulting ribozyme expression plasmids RzA, RzB and RzC were designed to cleave 386, 1,991 or 2,558 nucleotides downstream of the translation-initiation site in the ErbB-2 mRNA (Fig. 1a). Correct sequences of the ribozymes were verified by DNA sequencing, and specific catalytic ribozyme activity was demonstrated in *in vitro* cleavage assays (data not shown).

##### Cell lines, transfections and growth assays

Human ovarian cancer cells (SK-OV-3) were obtained from the ATCC (Manassas, VA) and maintained in culture at 37°C in 5% CO<sub>2</sub> using Iscove's modified Eagle's medium (IMEM; Life Technologies, Bethesda, MD) supplemented with glutamine and 10% heat-inactivated FBS (Biofluids, Rockville, MD). SK-OV-3 cells were transfected using Lipofectamine (Life Technologies). Briefly, cells at 50% to 70% confluence were incubated for 5 hr with 20 µg plasmid DNA mixed with 140 µl Lipofectamine in serum-free medium (Opti-MEM, Life Technologies) at 37°C in 5% CO<sub>2</sub>. The transfection medium was then replaced with normal growth medium and, 36 hr later, supplemented with G418 at 700 µg/ml for selection of stable integrants. Clonal cell lines were obtained by limited dilution of mass-transfected cell lines. To determine anchorage-dependent proliferation rates of differently transfected SK-OV-3 cell lines, 500 cells were plated in triplicate into 96-well plates and cell numbers quantified at different time points using a colorimetric assay based on cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Cell Proliferation Reagent WST-1; Boehringer-Mannheim, Mannheim, Germany). Studies of anchorage-independent growth of SK-OV-3 cells in soft agar were carried out as described (Fang *et al.*, 1992). Briefly, 10<sup>4</sup> cells in 0.35% agar (Difcoagar, Life Technologies) were layered on top of 1 ml of a solidified 0.6% layer in a 35 mm dish. The material to be tested was filter-sterilized, and EGF (Life Technologies) added with the 900 µl top layer to final concentrations ranging from 0.1 to 100 ng/ml. Growth media with 10% FBS were included in both layers. For studies of anchorage-dependent growth in methylcellulose (4,000 centipoise; Fisher, Pittsburgh, PA), a sterile 2.2% stock solution of methylcellulose was prepared. Briefly, 22 g of methylcellulose were dissolved in 500 ml of sterile water at 4°C and 500 ml of 2 × IMEM added. The solution was left in a cold room at 4°C for 24 hr until the methylcellulose was completely dissolved and the solution was of homogenous color and texture. The solution was then heated to 37°C, dispensed into

50 ml conical tubes and stored at -20°C. Cells were suspended at 5 × 10<sup>6</sup>/ml in normal growth medium containing growth factors and 10% FBS, if not otherwise indicated. The methylcellulose stock solution was warmed to 37°C and mixed with the cell solution to give a final concentration of 1% methylcellulose with cells at a density of approximately 2 × 10<sup>5</sup>/ml; 10 ml of the methylcellulose/cell suspension were then plated in triplicate into 100 mm Petri dishes and incubated at 37°C in 5% CO<sub>2</sub> for the indicated time points. To harvest the cells at the end of the experiments, methylcellulose/cell solution was diluted with 30 ml of pre-warmed 1 × PBS and cells were collected by centrifugation at 700 g for 5 min. If needed, a second washing step in 20 ml of 1 × PBS was performed to remove residual methylcellulose.

##### Northern blot analysis

Total cellular RNA was isolated by the RNA STAT-60 method (Tel-Test, Friendswood, TX), and 20 µg were separated and blotted as described (Fang *et al.*, 1992). Human cDNA probes specific for ErbB-2 (1.5 kb EcoRI fragment) or c-Fos (900 bp AccI fragment) were hybridized and the blots washed and exposed to film for 48 hr (Fang *et al.*, 1992). To correct for variability in loading, blots were stripped, reprobed with a glyceraldehyde-3 phosphate dehydrogenase (G3PDH; Clontech, Palo Alto, CA) cDNA probe and exposed to film for 6 hr. Relative band intensities were measured by phosphorimaging.

##### Fluorescence-activated cell sorting (FACS)

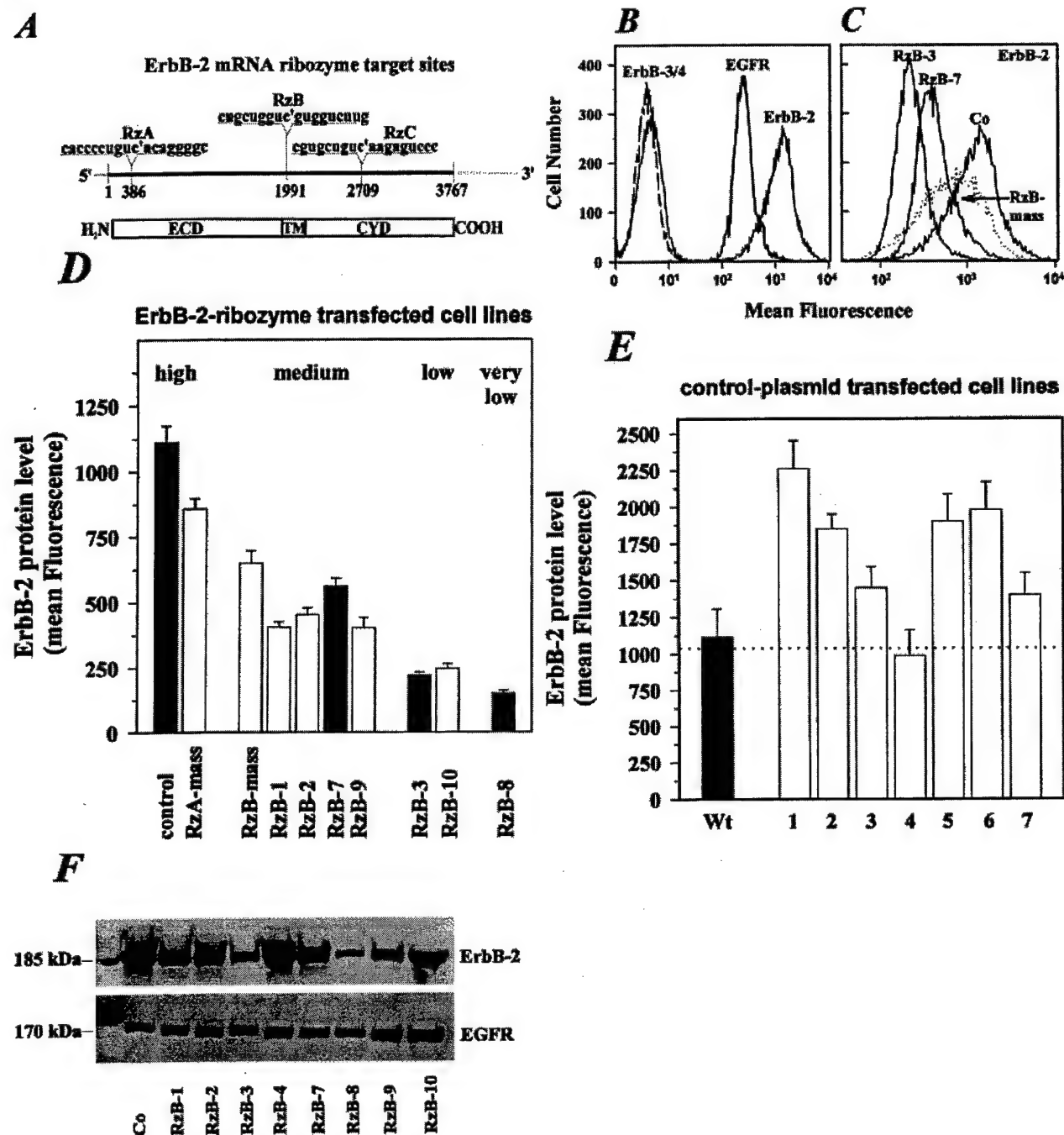
To quantitate the protein levels of the 4 ErbB receptor family members by FACS analysis, cells were trypsinized, washed once with growth medium containing serum and twice with PBS (Sigma, St. Louis, MO) and resuspended in PBS at 5 × 10<sup>5</sup> cells/100 µl. Cells were incubated for 30 min at 4°C with 1:50 dilutions of primary anti-human mouse MAbs specific for EGFR (clone EGFR1), ErbB-2 (clone 9G6.10), ErbB-3 (clone H3.90.6) or ErbB-4 (clone H4.77.16) (all antibodies obtained from Neomarkers, Fremont, CA). Cells were washed twice with PBS and incubated for 30 min at 4°C in the dark with a 1:200 diluted, FITC-labeled goat anti-mouse secondary antibody (Boehringer-Mannheim). After 2 final washes with PBS, the mean value of fluorescence intensity of 10<sup>4</sup> cells was determined by FACS analysis (FACStar plus; Becton Dickinson, Franklin Lakes, NJ). Unlabeled cells and cells labeled with secondary antibody alone served as negative controls.

##### Western blot analysis

Equal amounts of proteins from cellular extracts were separated by SDS-PAGE and proteins transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) for 2 hr at 150 mA/gel in 25 mM Tris (pH 8.3), 200 mM glycine, 20% methanol. Membranes were blocked for 1 hr in TBST [20 mM Tris (pH 7.5), 138 mM NaCl, 0.1% Tween 20], containing 5% non-fat dry milk and probed with 1:1,000 dilutions of polyclonal rabbit antibodies specific for ErbB-2 (RB-103-P, Neomarkers) or EGFR (gift from Dr. J. Pierce, NCI, NIH, Rockville, MD) for 1 hr at room temperature. Blots were then washed in TBST, 0.1% Tween 20 and incubated with a 1:5,000 dilution of a goat anti-rabbit secondary antibody coupled to horseradish peroxidase (Amersham, Aylesbury, UK) for 1 hr at room temperature. After additional washing in TBST, 0.1% Tween 20, bound antibody was visualized using an enhanced chemiluminescence reagent system (Amersham).

##### Immunoprecipitations

Cells were grown to approximately 70% confluence on plastic dishes, serum-starved for 24 hr and stimulated with EGF at 30 ng/ml for 10 min. Cell lysates were prepared as described (Souttou *et al.*, 1997): 1 mg of protein was pre-cleared with Protein A-Sepharose (EGFR) or Protein G-Sepharose (ErbB-2) and incubated overnight at 4°C with 4 µg of antibodies specific for EGFR (clone EGFR1) or ErbB-2 (clone 9G6.10). Immunocomplexes were captured with Protein A-Sepharose (EGFR) or Protein G-Sepharose (ErbB-2) at 4°C for 1 hr. Beads were then washed in IP



**FIGURE 1** – ErbB-2 mRNA ribozyme target sites and reduction of ErbB-2 expression in SK-OV-3 human ovarian cancer cells. (a) Top shows the position and sequences of the ribozyme target sites in ErbB-2 mRNA. Bold line represents the ErbB-2 mRNA coding region and dotted lines, the 5' and 3' untranslated regions. Ribozyme cleavage sites are numbered relative to the translation-start site in ErbB-2 mRNA. Lower part shows the main functional domains of the full-length ErbB-2 receptor protein relative to the mRNA coding region, with the extracellular domain (ECD), the transmembrane (TM) region and the cytoplasmic domain (CYD). (b) Representative FACS analysis of cell-surface expression of the 4 ErbB receptor family members in SK-OV-3 control cells. (c) Representative FACS analysis of ErbB-2 expression in control cells (Co) and some of the RZB ribozyme-expressing cell lines. (d) Mean fluorescence values  $\pm$  SD of FACS analysis of ErbB-2 expression in SK-OV-3 control cells, SK-OV-3 cells stably mass-transfected with ribozymes RZA (RZA-mass), RZB (RZB-mass) and a series of clonal cell lines derived from RZB-mass (RZB-1 to RZB-10). Cell lines are grouped in high (>70%), medium (>40%, <70%), low (>10%, <40%) and very low (<10%) levels of ErbB-2 expression. Filled bars indicate cell lines from each group used in functional studies (Figs. 2–5). (e) Mean fluorescence values  $\pm$  SD of FACS analysis of ErbB-2 expression in SK-OV-3 wild-type (wt) cells and a series of 7 clonal cell lines derived from SK-OV-3 wild-type cells transfected with empty control plasmid. (f) Western blot analysis of EGFR and ErbB-2 protein expression in total cellular lysates prepared from SK-OV-3 control cells (Co) and RZB-expressing SK-OV-3 cell lines (RZB-1 to RZB-10).

buffer and proteins eluted by boiling in SDS-PAGE sample buffer and subjected to electrophoresis and Western blotting.

#### Cell-cycle analysis

Cells were serum-starved for 24 hr and stimulated with EGF at 30 ng/ml for up to 72 hr. Cell-cycle phase analysis was performed using the Vindelov method for flow-cytometric DNA analysis. In brief, cells were trypsinized and  $1 \times 10^6$  cells resuspended in 0.1 ml of a citrate/DMSO buffer and stored at  $-80^\circ\text{C}$ . Nuclei were prepared and stained with propidium iodide and analyzed within 3 hr (FACStar plus).

#### Evaluation of apoptosis

Spontaneous apoptosis was evaluated combining annexin V-FITC labeling with flow-cytometric analysis according to the instructions of the manufacturer (Trevigen, Gaithersburg, MD).

### RESULTS

#### Ribozyme targeting of endogenous ErbB-2 expression in SK-OV-3 ovarian cancer cells

SK-OV-3 cells were chosen to study the effect of ErbB-2 depletion on EGF-mediated proliferation because of their restricted cell-surface expression pattern of ErbB receptor subtypes. Expression of all 4 ErbB receptor family members was analyzed by FACS. SK-OV-3 cells expressed high levels of EGFR and ErbB-2. In contrast, there was no detectable expression of ErbB-3 or ErbB-4 receptors (Fig. 1b), though the antibodies did detect low levels of ErbB-3 and ErbB-4 protein expression in T47D breast cancer cells (data not shown). Ribozyme targeting of ErbB-2 in SK-OV-3 cells was then applied to generate a panel of derivative cell lines that express different extents of residual ErbB-2. Expression vectors for hammerhead ribozymes, which are targeted against 3 different sites in the ErbB-2 mRNA (Fig. 1a), were generated. Ribozyme expression is under the control of a CMV promoter, and a polyadenylation signal enhances the stability of the short ribozyme transcripts. Efficacy and specificity of ribozyme transcripts were demonstrated in *in vitro* cleavage assays (data not shown). SK-OV-3 cells were then transfected with the ribozyme vectors or an empty control plasmid, and G418 drug selection was used to generate stable mass-transfected cell lines. The amount of residual ErbB-2 mRNA and protein expression was assessed by Northern blot, Western blot and FACS. The extent of ErbB-2 mRNA reduction (data not shown) correlated well with that of ErbB-2 protein depletion. The most active ribozyme construct, RzB, reduced ErbB-2 cell-surface expression by 50% (RzB-mass, Fig. 1c,d). The other 2 constructs were less efficient, with ErbB-2 reductions of 25% and 21% (Fig. 1d and data not shown, respectively). To achieve a more pronounced degree of ErbB-2 depletion, clonal derivative cell lines were generated from RzB-mass cells. Examples of representative FACS and Western blot analyses of ErbB-2 expression with some of the cell lines are shown in Figure 1c and f, respectively. The quantitation of ErbB-2 protein levels by FACS analysis of 8 clonal cell lines is depicted in Figure 1d. ErbB-2 protein expression was reduced by more than 90% in one cell line (RzB-8, Fig. 1d). The cell lines were then grouped according to ErbB-2 expression level into high, medium, low and very low ErbB-2-expressing categories; and representative cell lines (Fig. 1d, filled bars) were chosen for further functional studies. To control for the influence of clonal selection on ErbB-2 expression, clonal cell lines were generated from wild-type cells transfected with empty vector and ErbB-2 protein expression was measured by FACS analysis. Six of the 7 clonal control cell lines showed even higher ErbB-2 expression than wild-type cells (Fig. 1e), suggesting that ErbB-2 confers a growth advantage to SK-OV-3 cells and that clonal selection cannot be the reason for the ErbB-2 depletion in ribozyme-transfected cell lines. As an additional control for the specificity of the ribozyme effects on ErbB-2 expression, EGFR protein levels were analyzed. Western blots (Fig. 1f) and quantitative FACS analysis (data not shown) showed

no significant differences in EGFR protein expression between ribozyme-transfected and control cells.

#### Down-regulation of ErbB-2 expression inhibits spontaneous and EGF-mediated stimulation of anchorage-independent growth

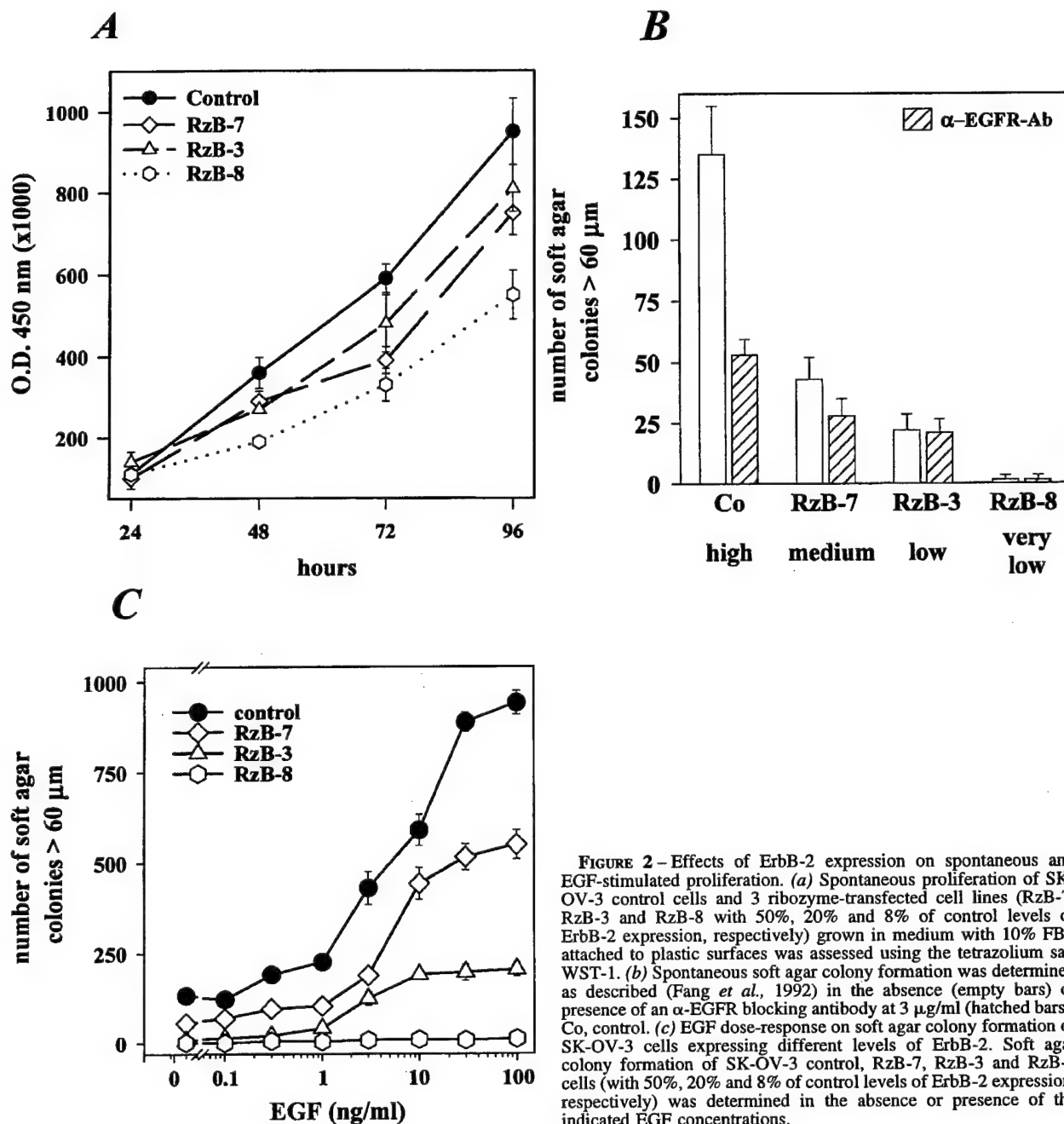
Previous studies with SK-OV-3 cells showed that ErbB-2 is rate-limiting for spontaneous soft agar colony formation and tumor growth in athymic nude mice (Juhl *et al.*, 1997). Here, we addressed the question of whether a gradual reduction in ErbB-2 expression would inhibit the EGF-mediated proliferative response in these cells. In a first set of experiments, the proliferation of the different cell lines grown in serum-containing medium attached to plastic surfaces was tested. Gradual ErbB-2 depletion was followed by inhibition of anchorage-dependent proliferation, especially in cells with ErbB-2 at very low levels (RzB-8, Fig. 2a). Since the overall effects of ErbB-2 depletion on anchorage-dependent proliferation were rather small, proliferation in soft agar was used as a more sensitive and demanding assay. Overall, spontaneous soft agar colony formation in a series of ribozyme-expressing and control cell lines correlated well with the respective ErbB-2 expression levels (Fig. 2b). Average colony formation reductions of 45% in medium (RzB-7), 70% in low (RzB-3) and >95% in very low (RzB-8) ErbB-2-expressing cell lines were found (Fig. 2b). An anti-EGFR blocking antibody reduced spontaneous colony growth by approximately 60% in control cells (Fig. 2b). This inhibition was lost in parallel with the gradual loss of ErbB-2 expression (Fig. 2b), suggesting that autocrine stimulation via the EGF pathway supports some of the spontaneous anchorage-independent proliferation.

In the next series of experiments, we asked whether the sensitivity ( $\text{EC}_{50}$ ) and responsivity (maximum response) to EGF is altered in cells with different residual levels of ErbB-2. As predicted from the expression pattern of ErbB receptors (Fig. 1b) and consistent with previous results (Daly *et al.*, 1997), soft agar growth of SK-OV-3 cells could not be stimulated by heregulin (data not shown). In contrast, EGF stimulated colony formation of SK-OV-3 control cells very effectively in a dose-dependent manner, with an  $\text{EC}_{50}$  of 3 ng/ml and a maximum 10-fold stimulation at 100 ng/ml (Fig. 2c, filled circles). The  $\text{EC}_{50}$  remained unchanged in ErbB-2-reduced cell lines; surprisingly, the maximum response was gradually reduced in parallel with the reduction of ErbB-2, and cell line RzB-8, with the lowest ErbB-2 level (8% of control), was completely refractory to EGF treatment (Fig. 2c). Next, we tested, in serum-starved cells grown on plastic dishes, whether these differences in EGF responsiveness could be attributed to differences in EGFR and ErbB-2 phosphorylation upon EGF stimulation. As expected, the ability of the different cell lines to autophosphorylate the EGFR was identical (Fig. 3a,b). However, when cell lysates were immunoprecipitated with an anti-ErbB-2 antibody and probed with an anti-phosphotyrosine-specific antibody ( $\alpha$ -P-Y), both constitutive and EGF-stimulated phosphorylations of ErbB-2 were reduced in parallel with the gradual loss of ErbB-2 expression (Fig. 3a). When cell lysates were immunoprecipitated with an anti-EGFR antibody and probed with an  $\alpha$ -P-Y, we observed, in addition to the major band at 170 kDa (EGFR), a second fainter band at approximately 185 kDa. This band most likely represented ligand-activated, phosphorylated EGFR protein migrating more slowly in the gel due to its increased phosphorylation. Together, these data suggest that the activated EGFR-ErbB-2 heterodimer is the rate-limiting receptor combination for the growth response and that the EGFR homodimer is not sufficient to induce the EGF effect on soft agar proliferation in SK-OV-3 cells.

#### EGF-induced c-Fos activation is decreased and spontaneous apoptosis is increased in ErbB-2-depleted SK-OV-3 cells

To test whether ErbB-2 depletion would inhibit EGF-mediated signal-transduction events, activation of c-Fos mRNA expression was assessed as a downstream marker in the EGF signal-transduction pathway. Cells were grown without serum in methylcellulose,

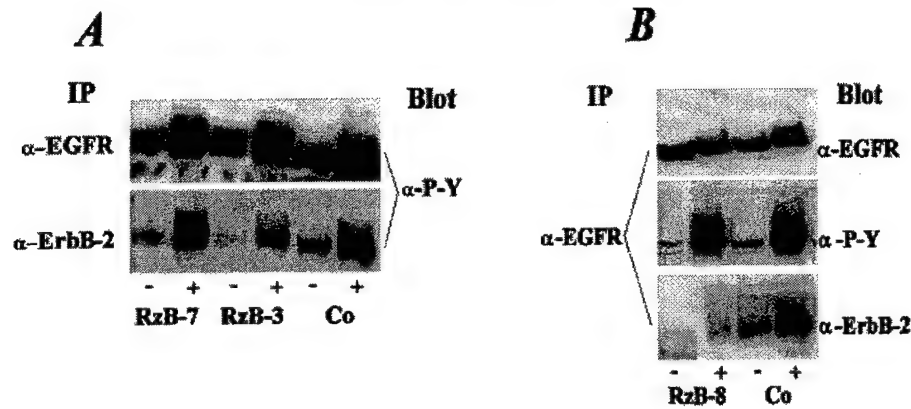




**FIGURE 2**—Effects of ErbB-2 expression on spontaneous and EGF-stimulated proliferation of SK-OV-3 control cells and 3 ribozyme-transfected cell lines (RzB-7, RzB-3 and RzB-8 with 50%, 20% and 8% of control levels of ErbB-2 expression, respectively) grown in medium with 10% FBS attached to plastic surfaces was assessed using the tetrazolium salt WST-1. (b) Spontaneous soft agar colony formation was determined as described (Fang *et al.*, 1992) in the absence (empty bars) or presence of an  $\alpha$ -EGFR blocking antibody at 3  $\mu$ g/ml (hatched bars). Co, control. (c) EGF dose-response on soft agar colony formation of SK-OV-3 cells expressing different levels of ErbB-2. Soft agar colony formation of SK-OV-3 control, RzB-7, RzB-3 and RzB-8 cells (with 50%, 20% and 8% of control levels of ErbB-2 expression, respectively) was determined in the absence or presence of the indicated EGF concentrations.

as described in Material and Methods, which provided conditions similar to soft agar but allowed easy harvesting of intact cells. EGF treatment up-regulated c-Fos mRNA expression approximately 20-fold after 30 min in control cells. This maximum response was only slightly altered in ErbB-2-depleted RzB-3 cells (Fig. 4a,b), and even RzB-8 cells with very low ErbB-2 expression showed a comparable c-Fos peak activation (data not shown). However, the duration of c-Fos activation was markedly reduced in ErbB-2-depleted cells. Control cells showed activation of c-Fos expression over 24 hr, whereas in RzB-3 cells c-Fos expression dropped to basal levels after only 2 hr of EGF treatment (Fig. 4a,b). This suggests that activation of the EGF homodimer is sufficient for the initial signaling response but that the sustained response requires activation of the ErbB-2-containing heterodimeric receptor.

Since tumor growth is the result of mitogenic and apoptotic events, we assessed the contribution of these pathways to the observed effects. As expected, in SK-OV-3 control cells, EGF treatment was followed by an increased fraction of cells entering S phase and EGF-mediated stimulation of cell-cycle progression was reduced in ErbB-2-depleted RzB-3 and RzB-8 cells (data not shown). The rate of spontaneous apoptosis was then assessed in cells grown in methylcellulose containing 10% FBS by annexin V-FITC binding, which is considered an early apoptotic event. Annexin V fluorescence was significantly increased in cells with reduced ErbB-2 levels (Fig. 5). Annexin V fluorescence (mean  $\pm$  SD) increased from  $5.7 \pm 1.1$  in control cells to  $7.1 \pm 1.8$  in RzB-7 cells,  $11.1 \pm 2.4$  in RzB-3 cells and  $25.5 \pm 3.1$  in RzB-8 cells (Fig. 5). These data provide a mechanism for the decrease of



**FIGURE 3** – EGF-induced tyrosine phosphorylation of EGFR and ErbB-2 receptors in SK-OV-3 control and ErbB-2-depleted cell lines. (a) Total cellular lysates from unstimulated (–) and EGF-stimulated (+, stimulation with EGF at 30 ng/ml for 10 min) SK-OV-3 control cells (Co) and RzB-7 and RzB-3 ribozyme-expressing cell lines (50% and 20% residual ErbB-2 expression, respectively) were subjected to immunoprecipitation (IP) with EGFR- (top panel) or ErbB-2- (bottom panel) specific antibodies. Tyrosine-phosphorylated proteins were detected by Western blotting with an  $\alpha$ -phosphotyrosine antibody ( $\alpha$ -P-Y). (b) Total cellular lysates from unstimulated (–) and EGF-stimulated (+) SK-OV-3 control cells (Co) and RzB-8 cells (8% residual ErbB-2 expression) were subjected to IP with an  $\alpha$ -EGFR antibody, and immunoreactive proteins were detected by Western blotting with antibodies specific for EGFR (top panel), phosphotyrosine (middle panel) or ErbB-2 (bottom panel).

spontaneous proliferation in anchorage-dependent (Fig. 2a) and anchorage-independent (Fig. 2b,c) growth assays of ErbB-2-depleted cell lines and show that constitutive ErbB-2 expression not only is rate-limiting for the mitogenic EGF response but also provides an important signal for cell survival.

#### DISCUSSION

Despite extensive knowledge on the phenomenology of ErbB-2 expression in human cancer, the exact molecular mechanisms underlying its oncogenic activity were unknown for a long time. Several groups have provided strong data that helped to define the concept of a complex signaling network of ErbB receptors and ligands (Alroy and Yarden, 1997; Salomon *et al.*, 1995). Within that signaling network, ErbB-2 has emerged as the preferred low-affinity subunit in all heterodimeric receptor combinations (Graus-Porta *et al.*, 1997). The ErbB-2-containing heterodimers have the strongest signal-transducing and growth-stimulating activities (Graus-Porta *et al.*, 1995; Karunakaran *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996). There appears to be a clear hierarchy of receptor dimer formation in response to different EGF-like or NDF/herregulin-like ligands (Alroy and Yarden, 1997). In most human tumors, various combinations of ErbB receptors are co-expressed (Salomon *et al.*, 1995). Which receptors or combinations are rate-limiting in distinct tumors is an important remaining question. Previous work in T47D breast cancer cells showed that abrogation of ErbB-2 cell-surface expression can diminish EGF- and NDF/herregulin-induced signal-transduction events (Graus-Porta *et al.*, 1995; Karunakaran *et al.*, 1996). Based on these data, we hypothesized that not only the signaling response but also the EGF-mediated stimulation of proliferation is dependent on ErbB-2 expression.

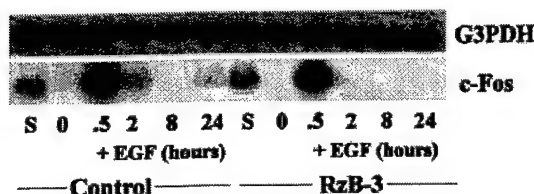
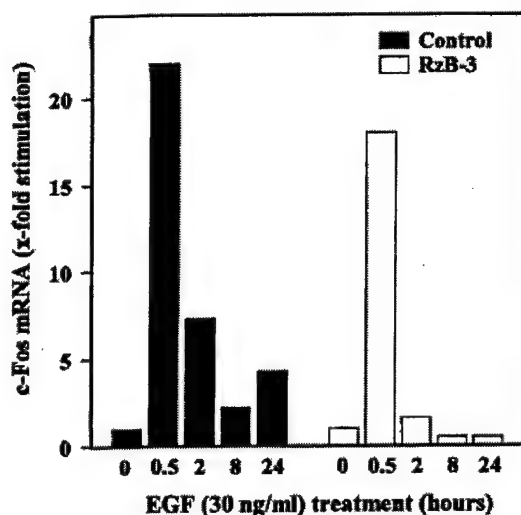
To study the role of ErbB-2 in EGF responsiveness, ErbB-2-targeted ribozymes were expressed in SK-OV-3 human ovarian cancer cells, which over-express EGF and ErbB-2 receptors but show no detectable cell-surface expression of the primary NDF/herregulin receptors ErbB-3 and ErbB-4 (Fig. 1b). We had previously shown that ErbB-2-targeted ribozymes down-regulate ErbB-2 expression very efficiently in SK-OV-3 cells (Juhl *et al.*, 1997; Czubyko *et al.*, 1997) and that ErbB-2 is rate-limiting for tumor growth in athymic nude mice (Juhl *et al.*, 1997). Here, the effect of a gradual reduction of ErbB-2 expression on EGF responsiveness was tested in a series of mass-transfected and clonal

derivative ribozyme-expressing cell lines (Fig. 1c,d). ErbB-2 expression levels correlated well with spontaneous soft agar colony formation (Fig. 2b,c). Exogenous addition of an anti-EGFR blocking antibody significantly reduced spontaneous colony formation (Fig. 2b), suggesting that growth of these cells depends on an autocrine loop driven by an EGF-like ligand and that ErbB-2 is the essential component in maintaining this loop. This autocrine function of ErbB-2 had also been suggested in SKBR-3 breast cancer cells using *ErbB-2* anti-sense targeting (Vaughn *et al.*, 1995) as well as in N87 gastric cancer cells through exogenous addition of inhibitory anti-ErbB-2 antibodies (Hurwitz *et al.*, 1995). This has been independently shown in a series of breast cancer and epithelial cancer cell lines through intracellular retention of ErbB-2 receptors using ErbB-2-targeted single-chain antibodies (Graus-Porta *et al.*, 1995).

However, the role of ErbB-2 on EGF-mediated proliferation remained unknown and was tested here by assessing EGF stimulation of colony formation in the aforementioned cell lines. Control SK-OV-3 cells were highly responsive to EGF, and the EGF response was absolutely dependent on ErbB-2 expression (Fig. 2c). This demonstrates that in SK-OV-3 cells the ErbB-2-containing heterodimer is the rate-limiting receptor combination and that the EGFR homodimer may not be sufficient to mediate the EGF proliferative response. In support of this, the ErbB-2-reduced cell lines showed normal EGFR but reduced ErbB-2 tyrosine phosphorylation upon EGF stimulation (Fig. 3a,b). This decrease was followed by a similar reduction in the duration of downstream signaling events such as c-Fos activation (Fig. 4a,b), which was reflected in the decreased amounts of stimulated cells entering S phase (data not shown). In addition, the percentage of cells spontaneously undergoing apoptosis was increased in ErbB-2-reduced cells (Fig. 5), providing a second mechanism for the inhibition of spontaneous as well as growth factor-induced proliferation.

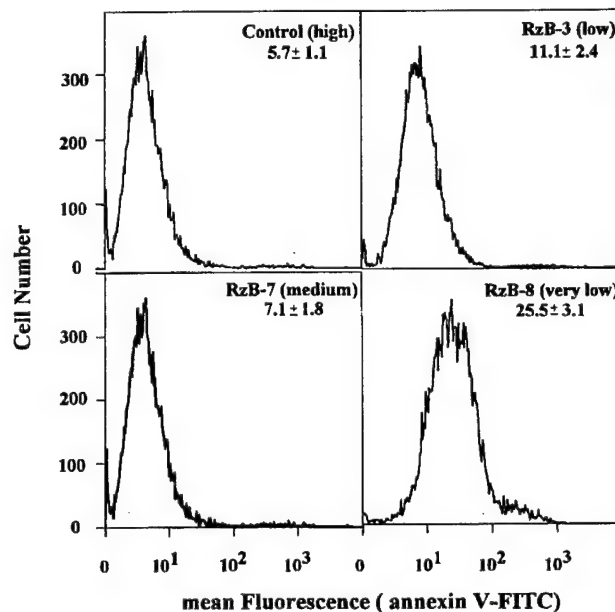
Previous studies in T47D breast cancer cells (Graus-Porta *et al.*, 1995; Karunakaran *et al.*, 1996) and 32D hematopoietic cells (Pinkas-Kramarski *et al.*, 1996) suggested a role for ErbB-2 in NDF/herregulin-mediated growth stimulation. In T47D cells, which express moderate amounts of all 4 ErbB receptors, loss of ErbB-2 cell-surface expression was accompanied by a reduction in EGF and NDF/herregulin binding (Graus-Porta *et al.*, 1995; Karunakaran *et al.*, 1996) as well as in the duration of downstream signaling events such as activation of MAP kinase and c-Jun kinase (Graus-Porta *et al.*, 1995; Karunakaran *et al.*, 1996). Although ErbB-2



**A****B**

**FIGURE 4** – EGF-mediated stimulation of c-Fos mRNA expression is reduced in ErbB-2-depleted SK-OV-3 cells. (a) SK-OV-3 control cells and RzB-3-expressing cells (20% residual ErbB-2 expression) were grown anchorage-independently in methylcellulose without serum and stimulated with EGF (30 ng/ml) for the indicated time points. Total cellular RNA (20 µg) was analyzed for c-Fos mRNA expression by Northern blotting. Blots were stripped and reprobed for G3PDH mRNA expression. (b) c-Fos mRNA expression levels were quantified relative to G3PDH mRNA levels, and values are expressed as x-fold stimulation (no EGF stimulation = 1).

expression was almost completely abrogated, spontaneous soft agar colony formation was unaffected and the weak stimulatory effect of NDF/hereregulin on colony formation was only mildly reduced (Graus-Porta *et al.*, 1995). However, EGF-mediated growth stimulation could not be tested in this model since wild-type T47D cells were completely unresponsive to EGF (Graus-Porta *et al.*, 1995). Thus, although T47D cells provided an excellent model to study the molecular mechanisms of the complex ErbB receptor signaling network, they were not useful for studying the effects of ErbB-2 reduction on growth factor-mediated tumor cell proliferation. This is probably due to the complex interplay of all 4 ErbB receptors and the confounding role of other growth-promoting pathways in these cells, such as steroid hormone-induced proliferation. Similarly, over-expression of various ErbB receptor combinations in 32D cells, which provide a completely negative background of endogenous ErbB receptors, demonstrated



**FIGURE 5** – Rate of spontaneous apoptosis is increased in cells with reduced levels of ErbB-2 expression. SK-OV-3 control cells and 3 RzB ribozyme-expressing cell lines with differently reduced ErbB-2 expression levels were grown anchorage-independently in methylcellulose containing 10% FBS, harvested as described and stained with annexin V-FITC as a marker for apoptosis. Numbers in the upper right corners indicate the respective mean annexin V-fluorescence levels (mean ± SD).

elegantly the potencies of the different ErbB receptor combinations and the superior signal-transducing activities of the ErbB-2-containing heterodimers (Graus-Porta *et al.*, 1995; Pinkas-Kramarski *et al.*, 1996). However, a rate-limiting role for ErbB-2 could not be demonstrated in this model since the EGF-activated EGFR homodimer on its own showed strong signal-transducing and proliferation-stimulating capabilities. The sensitivity and promiscuity of 32D cells to any transfected growth factor receptor limit the usefulness of this model in defining rate-limiting roles of particular receptor combinations. The complex interplay between the various ErbB receptors and ligands which are frequently over-expressed in human cancer raises the important physiological question of the role of these receptor interactions in different tumors. In this respect, ErbB-2 may act as a master regulator of the network, given that ErbB-2 serves as a partner and substrate of both NDF/hereregulin and EGF receptors.

Our data presented here show, in a human ovarian cancer cell line, that endogenously over-expressed ErbB-2 can be rate-limiting for EGF-induced tumor cell proliferation. These findings are probably relevant to many tumors that over-express ErbB-2 on the background of other ErbB receptors and further emphasize the use of selective gene targeting by ribozymes as an additional therapeutic strategy.

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## REFERENCES

- ALIMANDI, M., WANG, L.M., BOTTARO, D., FRANKEL, M., FEDI, P., TANG, C., LIPPMAN, M. and PIERCE, J.H., Epidermal growth factor and betacellulin mediate signal transduction through co-expressed ErbB2 and ErbB3 receptors. *EMBO J.*, **16**, 5608–5617 (1997).
- ALROY, I. and YARDEN, Y., The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand–receptor interactions. *FEBS Lett.*, **410**, 83–86 (1997).
- CARRAWAY, K.L., III and CANTLEY, L.C., A Neu acquaintance for ErbB3 and ErbB4: a role for receptor heterodimerization in growth signaling. *Cell*, **78**, 5–8 (1994).
- CZUBAYKO, F., DOWNING, S.G., HSIEH, S.S., GOLDSTEIN, D.J., LU, P.Y., TRAPNELL, B.C. and WELLSTEIN, A., Adenovirus-mediated transduction of ribozymes abrogates HER-2/neu and pleiotrophin expression and inhibits tumor cell proliferation. *Gene Ther.*, **4**, 943–949 (1997).
- DALY, J.M., JANNOT, C.B., BEERLI, R.R., GRAUS-PORTA, D., MAURER, F.G. and HYNES, N.E., Neu differentiation factor induces down-regulation and apoptosis of ErbB2-overexpressing breast tumor cells. *Cancer Res.*, **57**, 3804–3811 (1997).
- FANG, W.J., HARTMANN, N., CHOW, D., RIEGEL, A.T. and WELLSTEIN, A., Pleiotrophin stimulates fibroblasts and endothelial and epithelial cells and is expressed in human cancer. *J. biol. Chem.*, **267**, 25889–25897 (1992).
- GRAUS-PORTA, D., BEERLI, R.R., DALY, J.M. and HYNES, N.E., ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J.*, **16**, 1647–1655 (1997).
- GRAUS-PORTA, D., BEERLI, R.R. and HYNES, N.E., Single-chain antibody-mediated intracellular retention of ErbB-2 impairs Neu differentiation factor and epidermal growth factor signaling. *Mol. cell. Biol.*, **15**, 1182–1191 (1995).
- HURWITZ, E., STANCOVSKI, I., SELA, M. and YARDEN, Y., Suppression and promotion of tumor growth by monoclonal antibodies to ErbB-2 differentially correlate with cellular uptake. *Proc. nat. Acad. Sci. (Wash.)*, **92**, 3353–3357 (1995).
- JUHL, H., DOWNING, S.G., WELLSTEIN, A. and CZUBAYKO, F., HER-2/neu is rate-limiting for ovarian cancer growth: conditional depletion of HER-2/neu by ribozyme targeting. *J. biol. Chem.*, **272**, 29482–29486 (1997).
- KARUNAGARAN, D., TZAHAR, E., BEERLI, R.R., CHEN, X., GRAUS-PORTA, D., RATZKIN, B.J., SEGER, R., HYNES, N.E. and YARDEN, Y., ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO J.*, **15**, 254–264 (1996).
- LEWIS, G.D., LOFGREN, J.A., MCMURTREY, A.E., NUJENS, A., FENDLY, B.M., BAUER, K.D. and SLIWKOWSKI, M.X., Growth regulation of human breast and ovarian tumor cells by heregulin: evidence for the requirement of ErbB2 as a critical component in mediating heregulin responsiveness. *Cancer Res.*, **56**, 1457–1465 (1996).
- PIERCE, J.H., RUGGIERO, M., FLEMING, T.P., DI FIORE, P.P., GREENBERGER, J.S., VARTICOVSKI, L., SCHLESSINGER, J., ROVERA, G. and AARONSON, S.A., Signal transduction through the EGF receptor transfected in IL-3-dependent hematopoietic cells. *Science*, **239**, 628–631 (1988).
- PINKAS-KRAMARSKI, R., SOUSSAN, L., WATERMAN, H., LEVKOWITZ, G., ALROY, I., KLAPPER, L., LAVI, S., SEGER, R., RATZKIN, B.J., SELA, M. and YARDEN, Y., Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J.*, **15**, 2452–2467 (1996).
- SALOMON, D.S., BRANDT, R., CIARDIELLO, F. and NORMANNO, N., Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit. Rev. Oncol. Hematol.*, **19**, 183–232 (1995).
- SHAK, S., Overview of the trastuzumab (Herceptin) anti-HER2 monoclonal antibody clinical program in HER2 overexpressing metastatic breast cancer. Herceptin Multinational Investigator Study Group. *Semin. Oncol.*, **26** (Suppl. 12) 71–77 (1999).
- SLAMON, D.J., GODOLPHIN, W., JONES, L.A., HOLT, J.A., WONG, S.G., KEITH, D.E., LEVIN, W.J., STUART, S.G., UDVOVE, J. and ULLRICH, A., Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science*, **244**, 707–712 (1989).
- SOUTTOU, B., AHMAD, S., RIEGEL, A.T. and WELLSTEIN, A., Signal transduction pathways involved in the mitogenic activity of pleiotrophin. Implication of mitogen-activated protein kinase and phosphoinositide 3-kinase pathways. *J. biol. Chem.*, **272**, 19588–19593 (1997).
- TZAHAR, E., LEVKOWITZ, G., KARUNAGARAN, D., YI, L., PELES, E., LAVI, S., CHANG, D., LIU, N. and YAYON, A., ErbB-3 and ErbB-4 function as the respective low and high affinity receptors of all Neu differentiation factor/hergulin isoforms. *J. biol. Chem.*, **269**, 25226–25233 (1994).
- TZAHAR, E., PINKAS-KRAMARSKI, R., MOYER, J.D., KLAPPER, L.N., ALROY, I., LEVKOWITZ, G., SHELLY, M., HENIS, S., EISENSTEIN, M., RATZKIN, B.J., SELA, M., ANDREWS, G.C. and YARDEN, Y., Bivalence of EGF-like ligands drives the ErbB signaling network. *EMBO J.*, **16**, 4938–4950 (1997).
- VAUGHN, J.P., IGLEHART, J.D., DEMIRDJI, S., DAVIS, P., BABISS, L.E., CARUTHERS, M.H. and MARKS, J.R., Antisense DNA downregulation of the ERBB2 oncogene measured by a flow cytometric assay. *Proc. nat. Acad. Sci. (Wash.)*, **92**, 8338–8342 (1995).



## Expression of a truncated 100 kDa HER2 splice variant acts as an endogenous inhibitor of tumour cell proliferation

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Overexpression of the HER2 (neu/c-erbB-2) oncogene frequently coincides with an aggressive clinical course of certain human adenocarcinomas. Expression and secretion of aberrant HER2 splice variants has been reported in various cell lines and tissues and can interfere with the oncogenic HER2 activity. Here we demonstrate, using two different approaches, that expression of a truncated 100 kDa HER2 variant which encodes the extracellular domain of HER2 (HER-ECD) inhibits growth factor-mediated tumour cell proliferation. A HER2-ECD cDNA encoding the truncated variant was overexpressed in MCF7 breast cancer cells. HER2-ECD overexpression decreased spontaneous proliferation of MCF7 cells as well as heregulin-mediated soft agar colony formation. Concomitantly, heregulin-induced phosphorylation of HER4 as well as downstream activation of p44/p42 MAP-kinases was decreased. To confirm these data, ribozymes were targeted to the 3'-untranslated region of the 2.3 kb HER2-ECD mRNA which is spontaneously expressed in MKN7 gastric cancer cells. HER2-ECD-targeted ribozymes downregulated HER2-ECD expression and enhanced EGF-mediated soft agar colony formation of MKN7 cells. In parallel, EGF-induced activation of p44/p42 MAP-kinases and activation of c-Fos expression were increased in ribozyme-transfected MKN7 cells. Finally, in RT-PCR we found a trend towards a progressive loss of 2.3 kb HER2-ECD mRNA expression in more advanced gastric tumours. These data show that the HER2-ECD variant inhibits growth factor-mediated tumour cell proliferation suggesting an important role during the progression of human cancer. *Oncogene* (2001) 20, 2101–2111.

**Keywords:** HER2: c-erbB2 (neu); HER2-ECD; gastric cancer; breast cancer; ribozyme

### Introduction

The HER2 (neu, c-erbB-2) proto-oncogene encodes a 185 kDa transmembrane receptor-like tyrosine kinase that shares extensive homology with the other three members of the epidermal growth factor receptor family (HER receptor family) (Tzahar and Yarden, 1998). The HER receptor family establishes an extensive signalling network that plays a major role in processes like oncogenesis and embryogenesis (Tzahar and Yarden, 1998). HER2 overexpression has been observed in a variety of human epithelial tumours and has been linked to an unfavourable prognosis in patients with breast and ovarian cancer (Slamon *et al.*, 1987, 1989). Despite extensive efforts no high-affinity ligand or receptor-activating mutations of HER2 have been found (Tzahar and Yarden, 1998). Recently, HER2 has emerged as the preferred low-affinity subunit of all other HER receptors (Graus-Porta *et al.*, 1997; Tzahar *et al.*, 1996), and heterodimeric receptor combinations containing HER2 show superior signal-transducing and cell-growth stimulating capabilities (Graus-Porta *et al.*, 1995; Karunakaran *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996). Taken together, there is strong evidence that HER2 confers an autocrine growth advantage to tumour cells (Salomon *et al.*, 1995), and in addition, is responsible for increased paracrine responsiveness of tumour cells to stroma and/or tumour cell-derived EGF-like or heregulin-like growth factors (Hsieh *et al.*, 2000; Lewis *et al.*, 1996).

Many transmembrane growth factor and cytokine receptors have been reported to have soluble, ligand-binding receptor forms detectable in the conditioned media of tumour cells and in biological fluids such as serum and urine (Mosley *et al.*, 1989; Petch *et al.*, 1990; Zabrecky *et al.*, 1991). The widespread occurrence of soluble receptor forms from distinct proteins such as the interleukine-2-receptor (Rubin *et al.*, 1985), vascular endothelial growth factor (VEGF)-receptor (Kendall and Thomas, 1993), the HER1 (Petch *et al.*, 1990) HER2 (Zabrecky *et al.*, 1991) or HER3 receptor (Lee and Maible, 1998) suggests that these molecules may have important physiological roles. These soluble receptor proteins arise through proteolytic cleavage of

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membrane-anchored full-length receptors (Rubin *et al.*, 1985; Zabrecky *et al.*, 1991), and/or by alternative splicing or other gene rearrangements that usually produce novel transcripts which code for proteins containing parts of the ECD, but lack the transmembrane and cytoplasmic domains of the full-length receptor (Doherty *et al.*, 1999; Reiter and Maihle, 1996; Scott *et al.*, 1993).

Soluble truncated HER2 receptors which have been described *in vitro* and *in vivo* in a variety of tumour cell lines and tumour samples arise mainly through a proteolytic mechanism. However, there are at least two distinct truncated HER2-ECD proteins which are encoded by variant HER2 mRNA species. First, Scott *et al.* (1993) described a variant 2.3 kb HER2 mRNA which encodes the first 633 amino acids (aa) containing almost the entire HER2-ECD (subdomains I–IV). This truncated 100 kDa protein inhibited the growth-inhibitory effects of monoclonal HER2 antibodies presumably through an intracellular mechanism of action (Scott *et al.*, 1993). More recently, another soluble truncated 68 kDa HER2 protein has been described containing the first 340 aa of the HER2 protein (ECD-subdomains I and II) fused to a unique stretch of 79 aa which are encoded by a novel transcript through insertion of an intronic sequence (Doherty *et al.*, 1999). The secreted 68 kDa protein acts as an autocrine growth-inhibitor in tumour cells *in vitro* probably through interference with HER receptor dimerization (Doherty *et al.*, 1999). The potential physiological relevance of soluble truncated HER receptors are largely unexplored.

Here we show, that the 100 kDa HER2-ECD can act as a dominant-negative inhibitor of growth factor-mediated tumour cell proliferation. Doxycycline-regulated expression of the corresponding 2.3 kb HER-ECD cDNA in transfected MCF7 breast cancer cells showed HER2-ECD-mediated inhibition of spontaneous proliferation as well as inhibition of heregulin-mediated proliferation and signal transduction. Likewise, ribozyme-targeting of the endogenously expressed 2.3 kb HER2-ECD mRNA splice variant in MKN7 gastric cancer cells demonstrated reduction of HER2-ECD-mediated inhibition of EGF-mediated proliferation in soft agar and downstream signal-transduction. Furthermore, we provide data from some gastric tumour samples which suggest that reduced HER2-ECD expression is linked with a more aggressive phenotype, thus adding information towards the potential role of soluble truncated HER2 proteins in human tumours.

## Results

### *Genomic structure of the HER2 gene region containing the variant HER2-ECD sequence*

Since the aberrantly spliced HER2-ECD mRNA was originally sequenced from cDNA libraries only (Scott *et al.*, 1993), and since genomic sequence from this particular HER2 gene region is not yet available, we

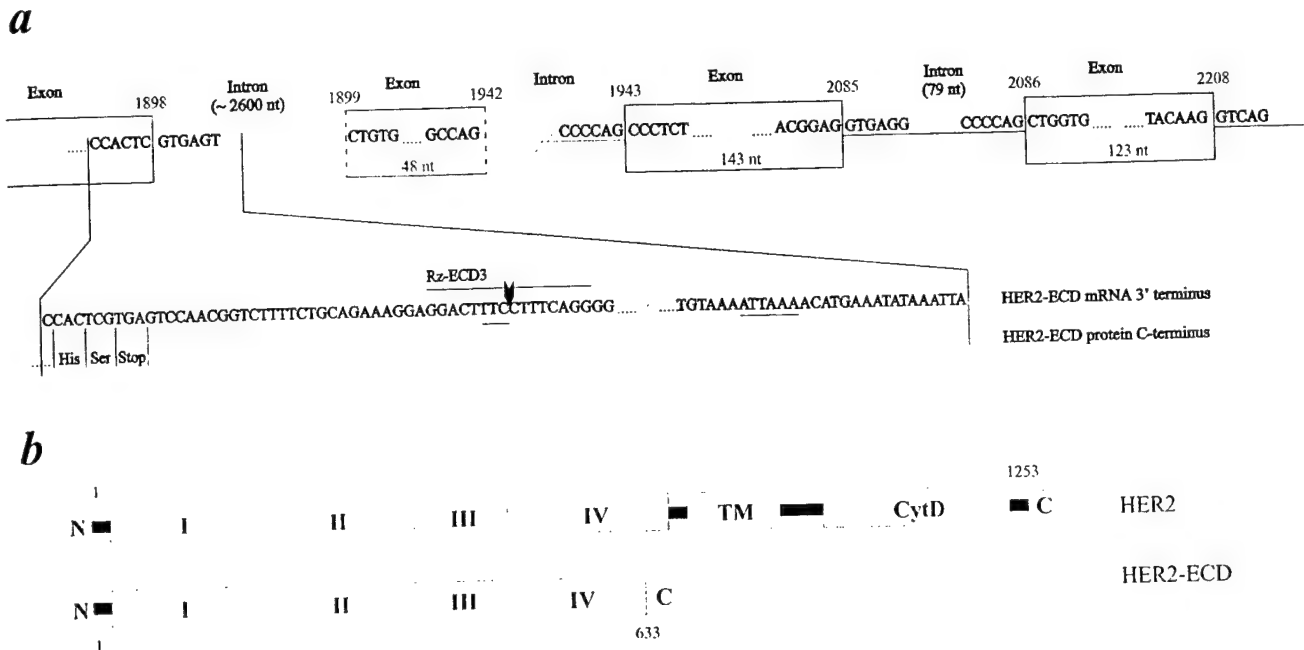
decided to use genomic sequencing in order to verify whether the divergent sequence is contiguous in the genome. Partial sequencing of a P1 phagemid clone confirmed the originally described exon/intron boundary, the in-frame stop codon and the novel polyadenylation signal of the divergent sequence followed by an intron of approximately 2.6 kb length (Figure 1a).

### *Regulated expression of the 100 kDa HER2-ECD variant in MCF7 breast cancer cells*

In order to study the biological role of the 100 kDa truncated HER2 protein, we chose MCF7 breast cancer cells as a model. MCF7 cells do not endogenously express the divergent HER2 mRNA; express only low levels of the full-length HER2 protein and respond very well to heregulin. We used a tetracycline-regulated expression system (tet-off system) as described (Juhl *et al.*, 1997) to generate MCF7 cells overexpressing the 2.3 kb HER2-ECD mRNA. Cell surface expression of full-length HER2, HER3 and HER4 receptors was measured by FACS analysis and remained unaltered in transfected MCF7 cells as compared to MCF7 wildtype cells (data not shown). Northern analysis of stably transfected MCF7-HER2-ECD cells showed that the 2.3 kb HER2-ECD mRNA was effectively expressed in the absence of doxycycline (Figure 2a; –dox) and that HER2-ECD mRNA expression could be repressed by more than 90% upon addition of doxycycline (Figure 2a,b; +dox). In addition, Western blot analysis from total cellular lysates revealed high expression levels of a single protein at approximately 100 kDa in the absence of doxycycline, representing the truncated HER2-ECD protein (Figure 2c; –dox vs +dox). Next we asked, whether the overexpressed HER2-ECD protein was also found in the conditioned media of transfected MCF7 cells. To that end a quantitative HER2 ELISA was used. In cellular lysates the truncated HER2-ECD protein was expressed at approximately 10-fold higher levels in the absence of doxycycline (Figure 2d; left panel) which correlated very well with the results from the Western analysis (Figure 2c). Significant amounts of HER2-ECD protein were also detected in the conditioned media (Figure 2d; right panel), demonstrating that the truncated HER2-ECD protein was effectively secreted in transfected MCF7 cells. The low abundance of full-length 185 kDa HER2 protein expression (Figure 2c) together with the marked dependence of the measured HER2 levels on the doxycycline treatment indicates that the HER2 ELISA detects truncated HER2-ECD protein that is secreted into the media rather than shedded HER2-ECD from membrane anchored full-length HER2 protein.

### *HER-ECD expression inhibits spontaneous as well as heregulin-mediated proliferation in MCF7 cells*

In the next series of experiments the impact of HER2-ECD overexpression on proliferation of MCF7 breast cancer cells was addressed by anchorage-dependent as



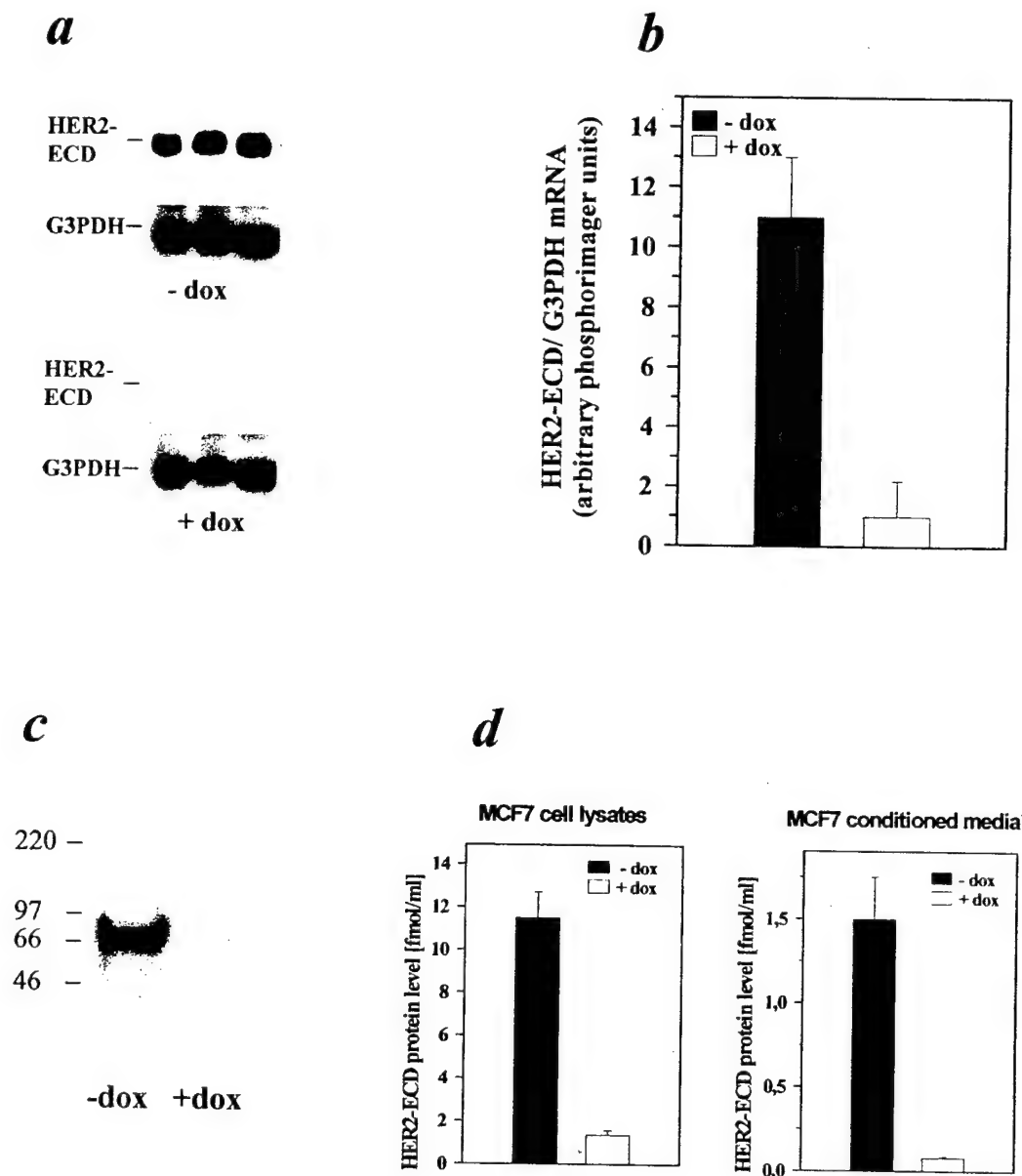
**Figure 1** Schematic diagram of the HER2 gene region containing the divergent HER2-ECD sequence and overview of the protein domain structure of the full-length and the truncated HER2 proteins. (a) Genomic DNA from a phagemid P1 clone containing the HER2 region of interest was partially sequenced and the sequence of three previously unknown HER2 exon/intron boundaries are shown. The boxed sequences contain exon sequence, and the intronic sequence of the adjacent exon/intron boundaries is shown on the horizontal lines when sequence information was available. The numbering on top of the exon borders represents the position in the full-length HER2 cDNA relative to the ATG in the translation start site. In the expanded brackets the divergent region of the 3'-terminus of the truncated HER2-ECD mRNA is shown with the retained 5'-splice donor site (GTGAG), the in-frame stop codon (TGA) and the polyadenylation signal (ATTTAA, underlined). The line on top of the HER2-ECD mRNA represents the target sequence of Rz-ECD3 hammerhead ribozyme designed to specifically cleave at the *TTC* in the 3'-untranslated region of the HER2-ECD mRNA. (b) The top diagram shows the schematic structure of the full-length (1253 amino acids) 185 kDa HER2 protein with the subdomains I, II, III and IV in the extracellular domain (ECD), the transmembrane domain (TM) and the cytoplasmic kinase domain (CytD). The diagram on the bottom illustrates the truncated HER2-ECD protein which is identical with the first 633 aa of the full-length HER2 protein and thus contains almost the entire HER2-ECD except for the most 3' 25 aa next to the TM-domain

well as anchorage-independent growth assays. HER2-ECD expression inhibited proliferation of MCF7 cells grown attached to plastic surfaces by approximately 50% (Figure 3a). Additionally, high levels of HER2-ECD expression significantly inhibited heregulin-mediated soft agar colony formation of MCF7 cells (Figure 3b; -dox vs +dox). Next we tested if the HER2-ECD effects on heregulin-mediated colony formation were paralleled by a similar inhibition of downstream signal-transduction events. As expected, stimulation of serum-starved cells with heregulin ( $10^{-9}$  M) increased the phosphorylation levels of the HER2 and the HER4 receptors (Figures 4a,b). A time course experiment showed that the heregulin-mediated increase of phosphorylation of the HER4 receptor was reduced and occurred with a time delay in HER2-ECD overexpressing cells (Figure 4b; -dox vs +dox). This reduction of HER4 receptor phosphorylation was followed by a similar inhibition of downstream signalling events such as activation of p44/p42 MAP-kinases (Figure 4c,d) and induction of c-Fos mRNA expression (data not shown). Since Scott *et al.* (1993) had previously proposed an intracellular mechanism of action based on interference of HER2-ECD with receptor internalization and recycling, we tested if the

recovery of cell surface expression of HER2 and HER3 receptors after heregulin-induced internalization was altered by HER2-ECD expression. There was no influence of HER2-ECD overexpression on the recovery time of cell surface expression of any of the HER2 and HER3 receptors in MCF7 cells (data not shown).

#### *Ribozyme-mediated downregulation of endogenous HER2-ECD expression in MKN7 gastric cancer cells*

As a second model we applied a ribozyme targeting strategy to specifically downregulate HER2-ECD mRNA expression in MKN7 gastric cancer cells. MKN7 cells were originally described as the only tumour cell line with very high expression levels of truncated as well as full-length HER2 proteins (Scott *et al.*, 1993). A hammerhead ribozyme was designed to specifically cleave in the unique 3'-untranslated region of the 2.3 kb HER2-ECD mRNA and stable mass-transfected HER2-ECD ribozyme expressing MKN7 cells were generated (MKN7/Rz-ECD3). Using this approach we expected to degrade the mRNA of the aberrantly spliced 2.3 kb HER2-transcript while leaving the mRNA of the full-length 4.5 kb HER2 transcript intact, thus altering the ratio of full-length



**Figure 2** Doxycycline-regulated overexpression of a truncated HER2-ECD cDNA coding for a 100 kDa HER2 protein variant (HER2-ECD) in MCF7 breast cancer cells. (a) Total RNA (20  $\mu$ g) from MCF7 cells stably expressing a 2.3 kb HER2-ECD mRNA under the control of a doxycycline-regulated promoter was analysed by Northern blotting for HER2-ECD mRNA expression in the absence (- dox) or presence (+ dox) of doxycycline (2  $\mu$ g/ml). (b) HER2-ECD mRNA expression was quantified relative to G3PDH by phosphorimaging. (c) MCF7 cell lysates were prepared as described and equal amounts of protein were separated on a denaturing 4–20% polyacrylamide gradient gel. The blots were probed with an anti-HER2-ECD-antibody which is able to detect truncated as well as full-length HER2 protein forms. (d) A quantitative HER2 ELISA assay was used to determine HER2-ECD protein levels in total cellular lysates and in conditioned media of transfected MCF7 cells grown in the absence or presence of doxycycline (2  $\mu$ g/ml). HER2 levels (mean  $\pm$  s.d.) were calculated according to a HER2 standard curve generated from recombinant HER2 as supplied by the manufacturer (data not shown)

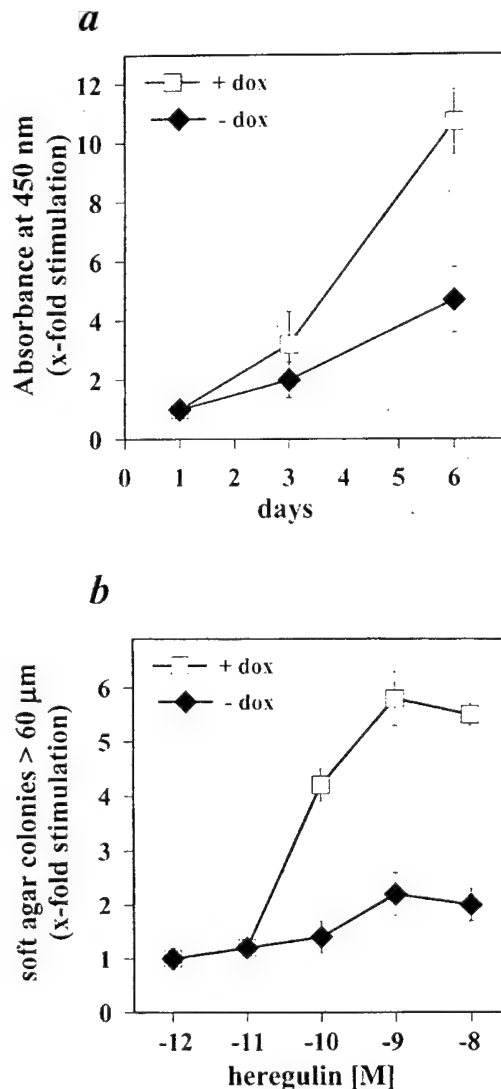
to truncated HER2 mRNA in MKN7 cells. Northern analysis (Figure 5a) demonstrated that Rz-ECD3 transfected MKN7 cells showed comparable levels of full-length HER2 mRNA while the truncated HER2-ECD mRNA was reduced by approximately 70% (Figures 5a,b). Western blot experiments from total cellular lysates showed a parallel reduction of the truncated 100 kDa HER2-ECD protein (data not shown), while FACS analysis demonstrated that there

was no change in cell surface expression levels of HER1, HER2 or HER3 receptors (data not shown).

#### Endogenous HER-ECD expression inhibits EGF-mediated proliferation and signal transduction in MKN7 cells

In order to test the biological significance of reduced expression of the truncated HER2-ECD in MKN7





**Figure 3** Spontaneous as well as heregulin-mediated proliferation are reduced in MCF7 cells overexpressing the HER2-ECD protein. (a) To determine anchorage-dependent proliferation rates, 250 cells were plated in triplicates into 96-well plates and grown in the absence (-dox) or presence (+dox) of doxycycline with addition of 10% serum. Cell numbers were quantified at different time points using a colorimetric assay and are presented as x-fold stimulation (mean  $\pm$  s.e.m. from three independent experiments) over background levels (background = cell numbers 24 h after plating (day 1) for each cell line). (b)  $10^4$  cells were plated in soft agar as described and grown in the absence (-dox) or presence (+dox) of doxycycline with addition of heregulin in the indicated concentrations. Soft agar colonies > 60  $\mu$ m were counted after 10 days of incubation and are presented as x-fold stimulation (mean  $\pm$  s.d. from three independent experiments) over background levels (background = no heregulin treatment)

cells, we tested the responsiveness to EGF in wildtype vs ribozyme-transfected cells. Soft agar colony formation of wildtype MKN7 cells was stimulated by EGF in a dose-dependent manner but there was only a slight approximately threefold maximum stimulation at 3 ng/ml of EGF (Figure 6). In contrast, ribozyme transfected MKN7 cells with reduced levels of HER2-ECD showed a significantly enhanced responsiveness

towards EGF with a maximum 10-fold stimulation. Next we tested in serum starved MKN7 cells whether these differences in EGF responsiveness could be attributed to changes in downstream signalling events. Serum-starved MKN7 cells with reduced levels of HER-ECD showed an approximately threefold higher induction of p44/p42 MAP-kinases after 30 min of EGF treatment as compared to wildtype cells (Figures 7a,b). In addition, ribozyme-transfected MKN7 cells showed an approximate twofold higher induction of c-Fos mRNA expression after 30 min of EGF treatment as compared to wildtype cells (Figures 7c,d).

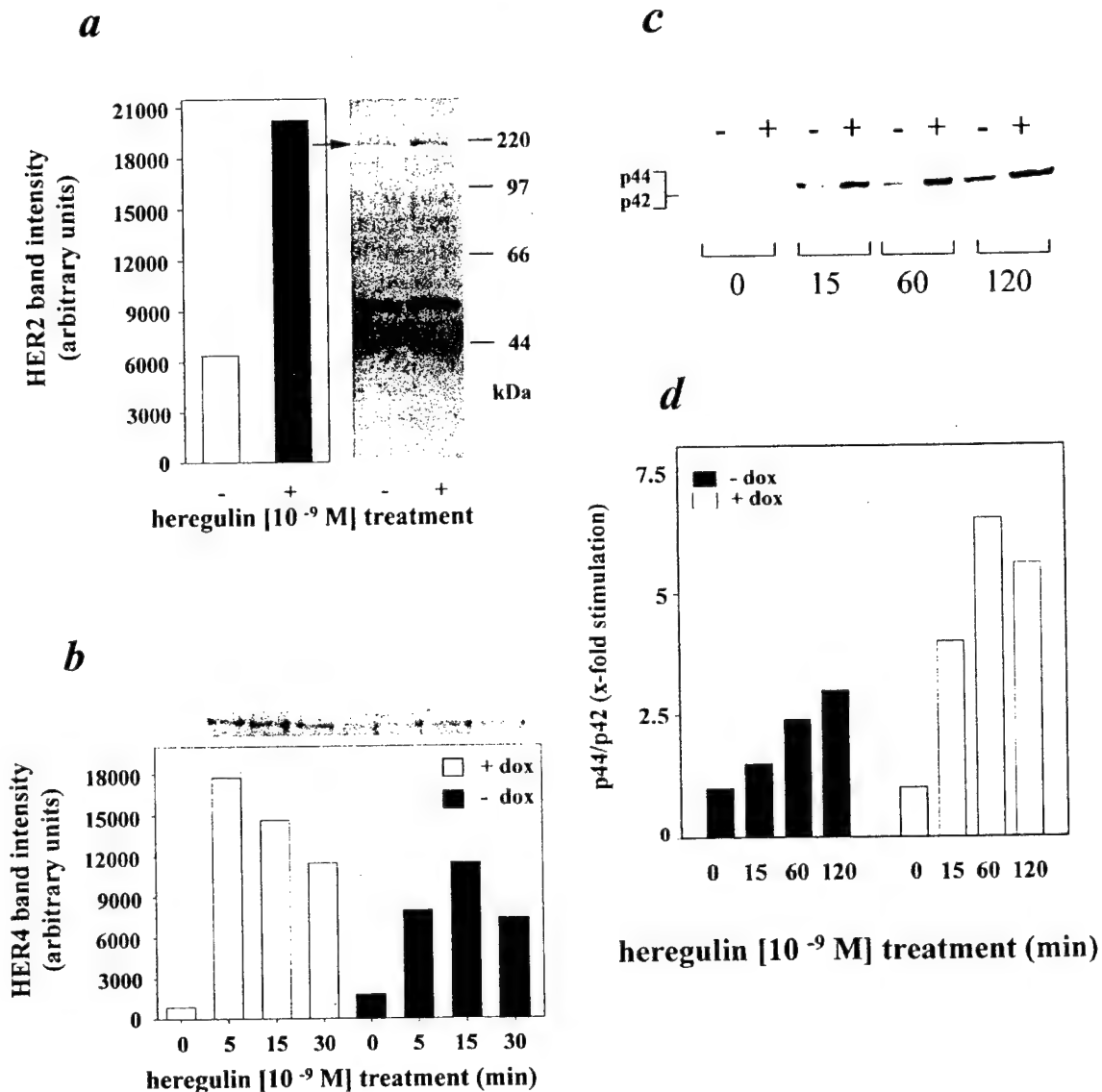
Since, as mentioned above, impairment of receptor recycling had been proposed as one possible mechanism of intracellular HER2-ECD action, we tested if recovery of HER1 and/or HER2 cell surface expression after discontinuation of EGF treatment was altered in ribozyme-transfected MKN7 cells. Again, as in MCF7 cells, the rate of HER1 and HER2 receptor internalization and time course of recovery of surface expression showed no significant differences between ribozyme-transfected and wildtype MKN7 cells (data not shown).

#### *HER-ECD expression is reduced in more advanced gastric tumours*

Since our data suggest that HER2-ECD expression can act as an inhibitor of tumour cell proliferation mediated by ligands of the HER receptor family, we asked whether this biological role of the truncated HER2 protein *in vitro* could also be observed in tumour samples *in vivo*. In a pilot study, expression of HER2-ECD as well as HER2 full-length mRNA was determined by RT-PCR. RT-PCR for the full-length HER2 transcript was positive in all 14 cases as compared to only seven out of 14 positive cases for the truncated HER2 transcript (Table 1). When the tumour samples were categorized according to TNM stages (I-IV) and biological grades (G1-G3) there was a trend towards reduced HER2-ECD expression in tumours with higher stages (IV) and biological grades (Table 1; three out of nine positive cases in G3 compared to four out of five in G2). It appears that loss of HER2-ECD expression could act as a positive selection event for gastric tumour cells.

#### **Discussion**

Alternative splicing of HER2 pre-mRNA generates at least three distinct mRNA's encoding the 185 kDa full-length membrane-spanning receptor that confers oncogenic activity and two truncated and soluble HER2-ECD proteins that are able to interfere with this activity. The first soluble HER2 receptor was described by Scott *et al.* (1993) as a 100 kDa HER2-ECD protein derived from a 2.3 kb HER2 transcript. Despite the presence of a canonical 5'-splice donor site, the sequence diverges in the truncated transcript at nt 1898 (with nt 1 counted as the translation start-site),



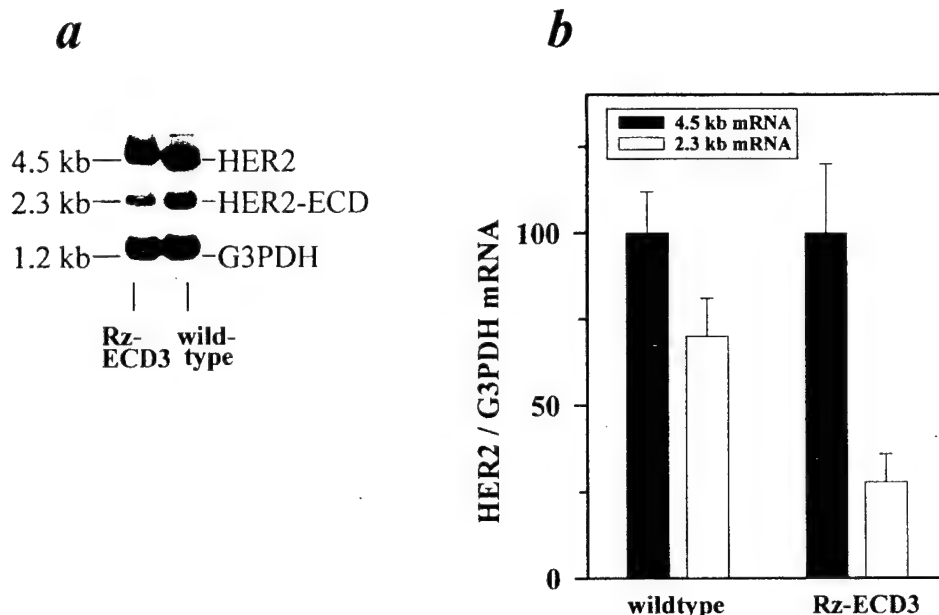
**Figure 4** Heregulin-mediated phosphorylation of HER2 and inhibition of heregulin-mediated induction of phosphorylation of HER4 and p44/p42 MAP-kinases in HER2-ECD overexpressing MCF-7 cell lines. MCF-7 cells grown in the absence (–dox) or presence (+dox) of doxycycline (2  $\mu$ g/ml) as indicated in the figures were serum starved for 48 h and then treated with heregulin ( $10^{-9}$  M) for the indicated time points. For Western blotting, total cellular lysates were prepared as described and equal amounts of proteins were separated on 4–20% polyacrylamide gels or immunoprecipitated and separated on denaturing 8% polyacrylamide gels. (a) After 10 min (+) vs no (–) heregulin stimulation, cellular lysates were immunoprecipitated with an anti-phosphotyrosine antibody. HER2 were detected with anti-HER2 antibodies (Ab 17) and HER-2-specific bands (right panel, arrow) were quantified by densitometry (left panel). (b) Cellular lysates were immunoprecipitated with anti-HER4 antibodies and phosphorylated HER4 was detected with an anti-phosphotyrosine antibody (upper panel). Bands were quantified by densitometry (bars). (c) Activated forms of p44/p42 MAP-kinases were detected with an antibody that is specific for the phosphorylated forms of p44/p42, and (d) were quantified by densitometry (bars represent the sum of p44 and p42 signal intensities)

revealing an exonic extension with an in-frame stop codon and a novel polyadenylation site. Here, sequencing of this particular HER2 gene-region confirmed the 5'-exon/intron boundary and the genomic contiguity. Thus it appears plausible that the truncated 2.3 kb HER2 mRNA arises from the absence of processing of this particular 5'-splice site resulting in a direct read-through of the contiguous intron sequence. Recently, another 2.6 kb HER2 mRNA variant has been described coding for a secreted

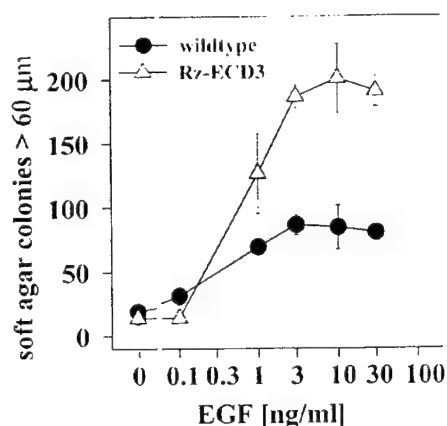
68 kDa protein (Doherty *et al.*, 1999). Both variants seem to be expressed only at low levels in tumour cells and tumour tissues, but are expressed at higher abundance in normal non-transformed cells.

Similar splice-site skipping of 3-introns has been described to occur in various other genes, including interleukin-5-receptor  $\alpha$ -subunits (Tavernier *et al.*, 1992), avian EGF-receptor (Flickinger *et al.*, 1992) and the VEGF-receptor (Kendall and Thomas, 1993). Production of alternatively transcribed soluble recep-





**Figure 5** Expression of endogenous HER2-ECD mRNA expression is reduced in ribozyme-transfected (Rz-ECD3) MKN7 gastric cancer cells. (a) Total RNA (20  $\mu$ g) from mass-transfected Rz-ECD3 or wildtype MKN7 cells was subjected to Northern blotting, and truncated 2.3 kb HER2-ECD mRNA, full-length 4.5 kb HER2 mRNA and G3PDH mRNA was detected by specific probes as described. (b) Full-length HER2 mRNA (filled bars, 4.5 kb transcript) and truncated HER2-ECD mRNA (open bars, 2.3 kb transcript) were quantified by phosphorimaging relative to G3PDH mRNA. The levels (mean  $\pm$  s.d.) of the truncated HER2-ECD mRNA are shown relative to their respective full-length HER2 mRNA expression levels

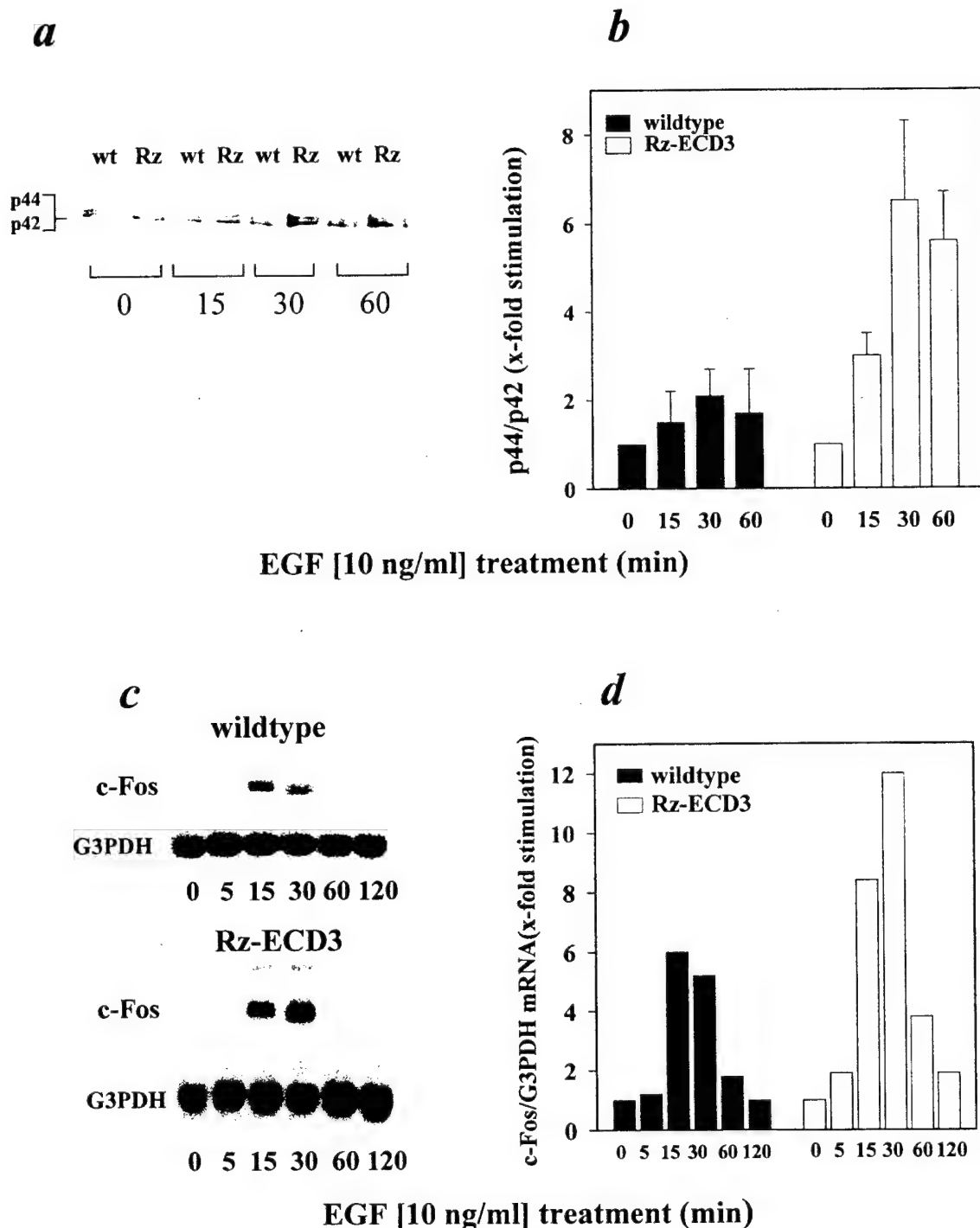


**Figure 6** EGF-mediated stimulation of soft-agar colony formation is increased in MKN7 cells transfected with HER-ECD-targeted ribozymes (Rz-ECD3).  $10^4$  cells were plated in soft agar as described and grown with the addition of EGF at the indicated concentrations. Soft agar colonies > 60  $\mu$ m were counted after 21 days of incubation (mean  $\pm$  s.d.)

tors could provide a mechanism for selectively and efficiently inhibiting cellular responsiveness to specific mitogenic and differentiation factors. These soluble receptors could exert their function through binding of their cognate ligands, or through interference with receptor dimerization and thus could act as specific high-affinity inhibitors *in vitro* and *in vivo*.

It was originally described that HER2-ECD expression abrogated the growth-inhibition mediated

by monoclonal anti-HER2-antibodies, and HER-ECD overexpression in transfected BT474 breast cancer cells which were selected in the presence of cytostatic doses of the anti-HER2-antibodies led to enhanced proliferation rates (Scott *et al.*, 1993). This in contrast to the decreased proliferation rates of transfected NIH3T3 cells as well as various tumour cells treated with recombinant 68 kDa HER2-ECD protein (Doherty *et al.*, 1999). However, it is possible that the enhanced proliferation of BT474 cells was an indirect effect due to the selection pressure exerted by the anti-HER2-antibody rather than a direct growth stimulatory effect exerted by the truncated 100 kDa HER2 variant. To address this issue, we used a tightly regulated expression system (tet-off system) to express the HER2-ECD in MCF7 cells. Using this approach, we observed that overexpression of the 100 kDa variant inhibited spontaneous as well as heregulin-mediated proliferation of MCF7 cells. Thus, since we used a tightly controlled expression system, it appears that in contrast to the previously described data in BT474 cells, it is more likely that the 100 kDa protein acts as an inhibitor of spontaneous tumour cell proliferation which is concordant with data obtained with the 68 kDa HER2 (Doherty *et al.*, 1999). The same role of the 100 kDa variant as an endogenous inhibitor of paracrine tumour cell proliferation was also observed in MKN7 gastric cancer cells that endogenously overexpress this truncated HER2-ECD form approximately in a 1:1 ratio with the full-length receptor. Ribozyme-trans-



**Figure 7** EGF-mediated stimulation of p44/p42 MAP-kinase activation and c-Fos mRNA expression is increased in MKN7 cells transfected with HER-ECD-targeted ribozymes (Rz-ECD3). (a) MKN7 wildtype cells and MKN7 cells transfected with the Rz-ECD3 ribozyme construct were serum-starved for 48 h and then treated with EGF (10 ng/ml) for the indicated time points. Total cellular lysates were prepared as described and equal amounts of proteins were separated on a denaturing 4–20% polyacrylamide gradient gel. Activated forms of p44/p42 MAP-kinases were detected with an antibody that is specific for the phosphorylated forms of p44/p42, and (b) were quantified by densitometry (filled bars (wildtype) and open bars (Rz-ECD3) represent the sum of p44 and p42 signal intensities). (c) In parallel experiments, total RNA was prepared from cells grown under the same experimental conditions and analysed by Northern blotting for c-Fos mRNA expression as described. c-Fos mRNA expression was quantified relative to G3PDH by phosphorimaging (d)

fectured MKN7 cells with downregulated levels of HER2-ECD transcripts displayed a much stronger

EGF-responsiveness in soft agar assays as compared to wildtype cells, thus confirming the role of the

**Table 1** Expression of the truncated 2.3 kb HER2-ECD mRNA as detected by RT-PCR is reduced in more advanced gastric cancer

	Truncated HER2-ECD mRNA	Full-length HER2 mRNA
Stage I	1/4	4/4
Stage II	2/2	2/2
Stage III	2/3	3/3
Stage IV	2/5	5/5
G1	—	—
G2	4/5	5/5
G3	3/9	9/9

100 kDa protein as an endogenous inhibitor of paracrine tumour cell proliferation in a different model system.

Based on the high and predominantly perinuclear expression levels of the 100 kDa protein, an intracellular mechanism of action had originally been proposed (Scott *et al.*, 1993), in which the 100 kDa HER2 form somehow interferes with the assembly of functional receptor dimers or inhibits the receptor recycling process after ligand-induced receptor internalization. In our studies we found no influence of HER2-ECD expression on ligand-induced receptor internalization rates or recovery of cell surface expression of HER1, HER2 or HER3 receptors in MCF7 or MKN7 cells. In contrast, HER-ECD expression reduced heregulin-mediated phosphorylation of HER4 receptors as well as downstream signalling events such as activation of p44/p42 MAP-kinases and c-Fos expression. These observations, together with our data that significant amounts of the 100 kDa HER2-ECD are secreted and thus can act extracellularly, suggest that the major mechanism of inhibitory action of the 100 kDa HER2-ECD variant is similar to the proposed model for the 68 kDa protein, where extracellular inhibition of HER2 receptor dimerization was demonstrated. Whatever the precise mechanism of soluble HER-ECD action is, the expression of soluble HER2 receptor forms could provide a selective pressure for reduced expression of truncated HER2-ECD proteins to overcome the growth-inhibition exerted by the truncated ECD forms in tumour cells. Indeed, when we tested for expression of the truncated and of the full-length HER-ECD transcripts in some gastric tumour samples, a tumour where HER2 expression is an independent marker of tumour progression and invasiveness (Allgayer *et al.*, 2000), we observed a trend towards a progressive loss of HER-ECD expression in more advanced tumours. Clearly, these results need to be confirmed in a larger number of tumour samples, especially since in 15 tumour samples from breast cancer patients HER-ECD expression was found to correlate with the development of micrometastases (Gebhardt *et al.*, 1998). It remains to be answered if the role of HER-ECD expression is dependent on the tumour type or other confounding variables.

It is conceivable that soluble HER2-ECD variants could be used as inhibitors of tumour growth *in vivo*.

On the other hand one needs to consider reports that high HER2-ECD serum levels in cancer patients were inversely correlated with the response to chemotherapy (Colomer *et al.*, 2000). Further studies concerning the distribution of HER-ECD expression in normal vs malignant tissues and during different stages of tumour development are needed. Likewise, the influence of HER2-ECD expression on established tumour treatments like chemotherapy, hormone treatment and radiation needs to be investigated in order to better understand and therapeutically exploit the important HER receptor network.

## Materials and methods

### Plasmids and generation of constructs

Plasmid expressing the tetracycline transactivating (tTA)/VP16 fusion protein (pUHG15.1) and the tTA/heptameric operator binding site (tet-O; pUHC13-3) were obtained from Dr Boujard (Heidelberg, Germany). The HER2-ECD expression plasmid (pTET) was derived from pUHC13-3 and modified as described (Schulte *et al.*, 1996). A 1.95 kb HER2-ECD *Bst*XI-fragment was cut from pw597.3a plasmid DNA containing a cDNA coding for the entire 2.3 kb HER2-ECD was cloned into the *Bst*XI site of pTET (pTET-HER2-ECD). The hammerhead ribozyme R<sub>z</sub>-ECD3 targeted specifically to the unique 3'-untranslated region (UTR) of the 2.3 kb HER2-ECD mRNA was designed to cleave 39 nt downstream of the unspliced exon/intron boundary and was cloned into the pRcCMV expression plasmid (Invitrogen, San Diego, CA, USA) as described (Czubayko *et al.*, 1994). In brief, the following R<sub>z</sub>-ECD3 ribozyme coding sense and antisense oligonucleotides: 5'-agctt**CCTGAAAGCTGATGAGTCCGTTAGGACGA**AAA-AGTCCAA-3' (sense) and 5'-agctt**AGGACTTTTTCGTCC-TAACGGACTCATCAGCTTTCAGGA**-3' (antisense) with small letters indicating *Hind*III-restriction site overhangs; bold capital letters showing HER2-ECD specific antisense regions; and italic capital letters indicating the hammerhead ribozyme core sequence, were annealed together and ligated into the *Hind*III-restriction site of pRcCMV.

### P1 library screening and HER2 intron sequencing

PCR screening of a human P1 Library (Genome Systems, St Louis, MO, USA) was performed using primers 5'-CTGCTGGTCTGTGTCTTGGG-3' (1881) and 5'-CCGTTTCCTGCAGCAGTCTCC-3' (2066rev). The P1 clone obtained from screening was subcloned (shotgun subcloning, Genome Systems, St Louis, MO, USA) in the pZERO-1 vector (Invitrogen, Carlsbad, CA, USA) using *Hind*III and *Xba*I. Subclones with an average insert size of 7–10 kb were screened with the same primers, and from positive subclones DNA was prepared as described (Genome Systems, St Louis, MO, USA). For sequencing into intron regions, various primers from exon regions were used. From the intronic sequence new primers were deduced to obtain additional intron sequence information.

### Cell lines, transfections and growth assays

MCF7 and MKN7 cell lines were maintained in culture at 37°C/5% CO<sub>2</sub> using Iscove's modified Dulbecco's medium (IMDM, PAA Laboratories, Cölbe, Germany) supplemented

with glutamine and 10% heat-inactivated foetal bovine serum (FBS, PAA Laboratories). MKN7 cells were transfected using LipofectAMINE (Life Technologies, Bethesda, MD, USA) as described (Juhl *et al.*, 1997) and stable mass-transfected cells were selected with G418 at 1 mg/ml. MCF7 cells stably expressing a tetracycline regulated 2.3 kb HER2-ECD mRNA were generated in a two-step transfection protocol as described (Juhl *et al.*, 1997). In a first step, MCF7 cells were transfected with pUHG15-1 plasmid DNA and pRc/CMV plasmid DNA (Invitrogen, San Diego, CA, USA) in a 10:1 ratio to provide G418 resistance. After selection for stable integrants in the presence of G418 at 0.7 mg/ml, individual tTA expressing clones were isolated with cloning rings and the clone (MCF7-600-10) that demonstrated the tightest tetracycline regulation was used for further transfections with the pTET/HER2-ECD plasmid. MCF7-600-10 cells were transfected with pTET HER2-ECD plasmid DNA mixed with pZero plasmid DNA (Invitrogen) in a 10:1 ratio to provide Zeocin resistance. Mass-transfected derivative cell pools were obtained after 6 weeks of selection with 0.4 mg/ml Zeocin in the continuous presence of 2 µg/ml doxycycline to repress transgene expression during the selection period.

To determine anchorage-dependent proliferation rates of transfected MCF7 and MKN7 cell lines, 250 to 1000 cells were plated in triplicates into 96-well plates and cell numbers were quantified at different time points using a colorimetric assay, which is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenases in viable cells (Cell Proliferation Reagent WST-1, Boehringer Mannheim, Mannheim, Germany). Studies of anchorage-independent growth of MCF7 and MKN7 cells were carried out as described (Czubayko *et al.*, 1994). Briefly,  $10^4$  cells in 0.35% agar (Difcoagar, Life Technologies) were layered on top of 1 ml of a solidified 0.6% agar layer in a 35 mm dish. EGF (Life Technologies; final concentrations ranging from 0.1 to 100 ng/ml) or heregulin (Neomarkers, Fremont, CA, USA; final concentrations ranging from 0.1 to 30 ng/ml) were added to the top layer mixture. Normal growth media with 10% FBS were included in both layers. In order to obtain better stimulation of colony formation of MCF7 cells by heregulin, the high levels of oestrogen-mediated spontaneous colony formation were reduced by a complete oestrogen receptor antagonist (ICI 182,780; Tocris, Bristol, UK) which was added to the top layer to a final concentration of  $10^{-8}$  M.

#### Northern analysis

Total cellular RNA was isolated with the RNA STAT-60 method (Tel-Test, Friedenswood, TX, USA), and 20 µg were separated and blotted as described (Czubayko *et al.*, 1994). Human cDNA probes specific for HER2 (1.5 kb *Eco*RI fragment from the HER2-ECD coding region) or c-Fos (900 bp *Acc*I fragment) were hybridized, washed and exposed to film for 24 h (HER2) or 72 h (c-Fos). To correct for variability in loading, blots were stripped, reprobed with a glyceraldehyde-3 phosphate dehydrogenase (G3PDH, Clontech, Palo Alto, CA, USA) cDNA probe and exposed to film for 6 h. Relative band intensities were measured by phosphorimaging.

#### Immunoprecipitation and Western analysis

For immunoprecipitation, 48 h serum-starved cells at 50–70% confluency were treated with heregulin prior to preparation of cell lysates as described (Soultou *et al.*, 1997). Briefly, 1 mg of protein was incubated overnight at

4°C with 20 µl of 4G10 anti-phosphotyrosine antibody coupled to agarose beads (UBI) or 2 µg of antibodies specific for HER4 (Neomarkers, Fremont, CA, USA; clone Ab 1). Immunocomplexes were captured with Protein G-Sepharose (HER4) at 4°C for 2 h. Beads were then washed in IP buffer and proteins eluted by boiling in SDS-PAGE sample buffer. Cells for Western analysis without prior immunoprecipitation were lysed in lysis buffer (2% SDS, 20% 2-mercaptoethanol, 10% glycerol). Western analysis was performed essentially as described (Aigner *et al.*, 2000). Briefly, equal amounts of proteins were separated on denaturing, reducing 8% or 4–20% polyacrylamide gels and transferred onto a nitrocellulose membrane (Protran BA 83, Schleicher & Schuell, Keene, NH, USA). After blocking with 3% BSA in TBS/0.05% Tween-20 (=TBST), the membrane was washed in TBST and probed in the same buffer with anti-HER1, 1:10000 (Neomarkers, Ab 3), anti-HER2, 1:100 (Neomarkers, Ab 3), anti-HER2, 1:400 (Neomarkers, Ab 17), 4G10 anti-phosphotyrosine antibody, 1:1000 (UBI), or anti-p44/p42 MAPK, 1:1000 (New England Biolabs, Beverly, MA, USA) which are specific for the phosphorylated forms of p44/p42-kinases, respectively, for 1 or 2 h as recommended by the supplier at room temperature. After extensive washing with TBST, bound antibodies were visualized by chemiluminescence using HRP-conjugated sheep anti-mouse antibodies and ECL detection reagents (Amersham, Buckinghamshire, UK). Band intensities were measured by densitometry (1D Image Analysis Software, Eastman Kodak Company, Rochester, NY, USA).

#### HER2 ELISA

Levels of intracellular and secreted HER2 were determined by ELISA (Oncogene, Cambridge, MA, USA) according to the manufacturer's protocol with modifications in the sample preparation.  $6 \times 10^5$  cells were grown for 48 h, the conditioned medium was collected, centrifuged, diluted 1:50 in sample diluent and directly used in the ELISA. The cells were washed twice with PBS and were lysed with 1.5 ml receptor buffer. After scraping, the lysate was transferred into a 2 ml vial, sonicated for 5 min and subjected to three freeze-thaw cycles to complete lysis. Fifty µl of the lysate were mixed with 10 µl antigen extraction buffer and incubated for 5 min. After centrifugation and determination of the protein concentration by BioRad assay, the supernatant was diluted in sample diluent to a final protein concentration of 5 µg/ml and measured in the ELISA.

#### Fluorescence-activated cell sorting (FACS)

Cell surface expression of the four HER receptor family members was quantitated by FACS-analysis as described (Hsieh *et al.*, 2000). Briefly, cells were incubated for 30 min at 4°C with 1:50 dilutions of primary anti-human mouse monoclonal antibodies specific for HER1 (Clone EGFR1; Neomarkers), HER2 (Clone 9G6.10; Neomarkers), HER3 (Clone H3.90.6) or HER4 (Clone H4.77.16), and labelled with a 1:200 diluted FITC-labelled goat anti-mouse secondary antibody (Boehringer Mannheim). The mean value of fluorescence intensity of 10 000 cells was determined by FACS (FACStar plus; Becton Dickinson, Franklin Lakes, NJ, USA).

#### RT-PCR

Total RNA from frozen gastric tumour samples was prepared and 2 µg of RNA were included in each RT-PCR reaction.

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Random-primed (for detection of full-length HER2-transcripts) or oligo(dT)-primed (for detection of HER2-ECD transcripts) cDNA's were generated using Superscript reverse transcriptase (Life Technologies) and specific fragments were amplified using Taq Polymerase (Life Technologies) with a set of primers specific for either of the two HER2 mRNA's (full-length HER2, forward primer: 5'-AGGGAGTATGTGAATGCC-3', and reverse primer: 5'-GGCCACTGGAATTTTCAC-3'; truncated HER2-ECD, forward primer: 5'-AGGGAGTATGTGAATGCC-3', and reverse primer: 5'-CCTGAAAGAAAGTCCTCC-3'). After amplification, the specific fragments of 290 nt (HER2-ECD) or 566 nt (full-length HER2) were separated and visualized in 1% agarose gels. In addition, a fragment specific for G3PDH mRNA was co-amplified in each sample to control for equal loading.

## References

- Aigner A, Malerczyk C, Houghtling R and Wellstein A. (2000). *Growth Factors*, **18**, 51–62.
- Allgayer H, Babic R, Gruetzner KU, Tarabichi A, Schildberg FW and Heiss MM. (2000). *J. Clin. Oncol.*, **18**, 2201–2209.
- Colomer R, Montero S, Lluch A, Ojeda B, Barnadas A, Casado A, Massuti B, Cortes-Funes H and Lloveras B. (2000). *Clin. Cancer Res.*, **6**, 2356–2362.
- Czubayko F, Riegel AT and Wellstein A. (1994). *J. Biol. Chem.*, **269**, 21358–21363.
- Doherty JK, Bond C, Jardim A, Adelman JP and Clinton GM. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 10869–10874.
- Flickinger TW, Maible NJ and Kung HJ. (1992). *Mol. Cell. Biol.*, **12**, 883–893.
- Gebhardt F, Zanker KS and Brandt B. (1998). *Biochem. Biophys. Res. Comm.*, **247** (2), 319–323.
- Graus-Porta D, Beerli RR, Daly JM and Hynes NE. (1997). *EMBO J.*, **16**, 1647–1655.
- Graus-Porta D, Beerli RR and Hynes NE. (1995). *Mol. Cell. Biol.*, **15**, 1182–1191.
- Hsieh SS, Malerczyk C, Aigner A and Czubayko F. (2000). *Int. J. Cancer*, **86**, 644–651.
- Juhl H, Downing SG, Wellstein A and Czubayko F. (1997). *J. Biol. Chem.*, **272**, 29482–29486.
- Karunagaran D, Tzahar E, Beerli RR, Chen X, Graus-Porta D, Ratzkin BJ, Seger R, Hynes NE and Yarden Y. (1996). *EMBO J.*, **15**, 254–264.
- Kendall RL and Thomas KA. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 10705–10709.
- Lee H and Maible NJ. (1998). *Oncogene*, **16**, 3243–3252.
- Lewis GD, Lofgren JA, McMurtrey AE, Nuijens A, Fendly BM, Bauer KD and Sliwkowski MX. (1996). *Cancer Res.*, **56**, 1457–1465.
- Mosley B, Beckmann MP, March CJ, Idzerda RL, Gimpel SD, VandenBos T, Friend D, Alpert A, Anderson D and Jackson J. (1989). *Cell*, **59**, 335–348.
- Petch LA, Harris J, Raymond VW, Blasband A, Lee DC and Earp HS. (1990). *Mol. Cell. Biol.*, **10**, 2973–2982.
- Pinkas-Kramarski R, Soussan L, Waterman H, Levkowitz G, Alroy I, Klapper L, Lavi S, Seger R, Ratzkin BJ, Sela M and Yarden Y. (1996). *EMBO J.*, **15**, 2452–2467.
- Reiter JL and Maible NJ. (1996). *Nucleic Acids Res.*, **24**, 4050–4056.
- Rubin LA, Kurman CC, Fritz ME, Biddison WE, Boutin B, Yarchoan R and Nelson DL. (1985). *J. Immunol.*, **135**, 3172–3177.
- Salomon DS, Brandt R, Ciardiello F and Normanno N. (1995). *Critical Reviews in Oncology and Hematology*, **19**, 183–232.
- Schulte AM, Lai S, Kurtz A, Czubayko F, Riegel AT and Wellstein A. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 14759–14764.
- Scott GK, Robles R, Park JW, Montgomery PA, Daniel J, Holmes WE, Lee J, Keller GA, Li WL and Fendly BM. (1993). *Mol. Cell. Biol.*, **13**, 2247–2257.
- Slamon DJ, Clark GM, Wong SG, Levin W, Ullrich A and McGuire RL. (1987). *Science*, **235**, 177–182.
- Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J and Ullrich A. (1989). *Science*, **244**, 707–712.
- Souttou B, Ahmad S, Riegel AT and Wellstein A. (1997). *J. Biol. Chem.*, **272**, 19588–19593.
- Tavernier J, Tuypens T, Plaetinck G, Verhee A, Fiers W and Devos R. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 7041–7045.
- Tzahar E, Waterman H, Chen X, Levkowitz G, Karunagaran D, Lavi S, Ratzkin BJ and Yarden Y. (1996). *Mol. Cell. Biol.*, **16**, 5276–5287.
- Tzahar E and Yarden Y. (1998). *Biochim. Biophys. Acta*, **1377**, M25–M37.
- Zabrecky JR, Lam T, McKenzie SJ and Carney W. (1991). *J. Biol. Chem.*, **266**, 1716–1720.

# HER-2/neu Is Rate-limiting for Ovarian Cancer Growth

## CONDITIONAL DEPLETION OF HER-2/neu BY RIBOZYME TARGETING\*

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**Amplification and overexpression of the *HER-2/neu* proto-oncogene frequently coincide with an aggressive clinical course of certain human adenocarcinomas. To assess whether *HER-2/neu* plays a rate-limiting role in ovarian cancer, we used human SK-OV-3 ovarian cancer cells as a model. We applied a conditional mRNA depletion strategy of *HER-2/neu* with anti-*HER-2/neu*-targeted hammerhead ribozymes expressed under the control of a tetracycline-regulated promoter system. In these ovarian cancer cells, we reduced *HER-2/neu* mRNA, protein expression, and tumor growth in nude mice by transfection with *HER-2/neu*-targeted ribozymes and generated cell lines expressing different levels of *HER-2/neu*. Expression of the most effective ribozyme (Rz3) quenched *HER-2/neu* mRNA levels by >90%. Concomitantly, fluorescence-activated cell sorting analysis revealed that expression of the *HER-2/neu*-encoded surface glycoprotein was almost completely abrogated. In nude mice, tumor growth was dramatically inhibited in the *HER-2/neu*-depleted Rz3-expressing SK-OV-3 cells. Furthermore, already established tumors started to regress when Rz3 expression was activated midstream by withdrawal of the tetracycline treatment. This study supports the thesis that *HER-2/neu* can be rate-limiting for the malignant phenotype of ovarian cancer in a gene dose-dependent manner.**

The *HER-2/neu* proto-oncogene belongs to the epidermal growth factor receptor family and has been implicated in malignant transformation (reviewed in Ref. 1). *HER-2/neu* can be activated by at least three different genetic mechanisms including point mutation (2), gene amplification (3), and overexpression (4). These observations are relevant to human cancer because amplification and/or overexpression has been observed in 20–30% of adenocarcinomas of the breast, ovary, lung, and stomach (reviewed in Ref. 5). Moreover, overexpression has been linked to an unfavorable prognosis in patients with breast (3) and ovarian (6) cancer.

Beyond this coincidence, it has been difficult to demonstrate directly that *HER-2/neu* is rate-limiting for tumor progression. One reason for this lack of understanding is that no ligand for *HER-2/neu* has been found, and *HER-2/neu* is now viewed

merely as a signal-transducing subunit of epidermal growth factor and new differentiation factor/herregulin receptors (7). Depending on the cellular context, *HER-2/neu*-targeted antibodies can thus cause activating and inhibitory effects, which make it difficult to dissect the precise role of *HER-2/neu*. Recently, with a novel approach using single-chain antibodies that suppress cell-surface expression of *HER-2/neu* by retention in the endoplasmic reticulum, it was shown that reduction of *HER-2/neu* reversed the transformed phenotype of *HER-2/neu*-transfected NIH/3T3 cells (8) and impaired growth factor signaling in T47D breast cancer cells (7, 9). Here we utilize an independent approach by cleaving the *HER-2/neu* mRNA with specific ribozymes and thus deplete cells of the endogenous gene product. With this approach, the effects of a functional knockout can be studied in model cell lines, and thus, the contribution of a particular gene product delineated (reviewed in Ref. 10; see also Refs. 11–14).

A major obstacle in achieving a constitutive ribozyme-mediated *HER-2/neu* depletion in stably transfected cells is that *HER-2/neu* expression may provide a growth advantage, thus making the selection of low expressing cell populations difficult. To circumvent this potential problem, we expressed hammerhead ribozymes under the control of a tetracycline-regulated promoter (15) to evaluate the effect of a conditional *HER-2/neu* depletion on *in vitro* and *in vivo* ovarian cancer cell growth. As a model, we used human SK-OV-3 ovarian cancer cells, which overexpress *HER-2/neu* due to a gene amplification event (16). We generated stably mass-transfected derivative cell lines that express anti-*HER-2/neu*-targeted ribozymes. Ribozyme expression almost completely abrogated *HER-2/neu* mRNA and protein expression, which resulted in a dramatic inhibition of tumor growth in nude mice. Furthermore, tumors that had been established in the absence of ribozyme expression started to regress when ribozyme expression was activated *in vivo*.

### EXPERIMENTAL PROCEDURES

**Plasmids and Generation of Constructs**—Plasmids expressing the tetracycline transactivator (tTA)<sup>1</sup>/VP16 fusion protein (pUHG15-1 (15)) and the heptamerized tetracycline operator sequence (tet-O; pUHC13-3 (15)) were obtained from Dr. Bujard (Zentrum für Molekulare Biologie der Universität Heidelberg, Heidelberg, Germany). The ribozyme expression plasmid (pTET) was derived from pUHC13-3 and modified as described (17). The *HER-2/neu*-targeted hammerhead ribozymes Rz3 and Rz4 were designed and cloned as described (18). In brief, the following ribozyme coding sense and antisense oligonucleotides were annealed together and ligated into the *Hind*III restriction site of pTET: Rz3, 5'-agcttCCTGAAAGCTGATGACTCCGTTAGGACG\*AAAAAGT-CCTa-3' (sense) and 5'-agcttAGGACTTTTTC\*GTCCTAACGGACTC-ATCAGCTTTCAGGA-3' (antisense); and Rz4, 5'-agcttCAAGACCAC-CTGATGAGTCCGTTAGGACGAAACCAGCAGa-3' (sense) and 5'-

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<sup>1</sup> The abbreviations used are: tTA, tetracycline transactivator; FACS, fluorescence-activated cell sorting; tet-O, heptamerized tetracycline operator sequence.



agcttCTGCTGGTTTCGCTCAACGGACTCATCAGGTGGTCTTG-a-3' (antisense) (with lower-case letters indicating *Hind*III restriction site overhangs, boldface upper-case letters showing HER-2/neu-specific antisense regions, and underlined upper-case letters indicating the hammerhead ribozyme core sequence). The resulting ribozyme expression plasmid (pTET/Rz3) contains HER-2/neu-specific antisense flanking regions of 8 nucleotides on both ends of the 22-nucleotide catalytic hammerhead ribozyme core sequence that target it to a central region in the HER-2/neu RNA just downstream of the transmembrane region of the protein. In addition, a control ribozyme was constructed (pTET/Rz3mu) that lacks catalytic activity due to a single base pair mutation in the ribozyme core region (G to T exchange; \* indicates the position of the mutated G). The ribozyme pTET/Rz4 contains HER-2/neu-specific antisense flanking regions of 9 and 8 nucleotides on the 5'- and 3'-ends, respectively, of the 22-nucleotide catalytic ribozyme core that target R24 to its cleavage site 1991 nucleotides downstream of the translation initiation site in the HER-2/neu RNA. Correct sequences of the ribozymes were verified by DNA sequencing, and specific catalytic ribozyme activity was demonstrated in *in vitro* cleavage assays as described (18).

**Cell Lines and Transfections**—Human ovarian cancer cells (SK-OV-3) were obtained from American Type Culture Collection and were maintained in continuous culture at 37 °C in 5% CO<sub>2</sub> using Iscove's modified Eagle's medium (Life Technologies, Inc.) supplemented with glutamine, 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. SK-OV-3 cells were transfected using LipofectAMINE (Life Technologies, Inc.). Briefly, cells at 50–70% confluence were incubated for 5 h with plasmid DNA mixed with LipofectAMINE (7 µl of LipofectAMINE/1 µg of plasmid DNA) in serum-free medium (Opti-MEM, Life Technologies, Inc.) at 37 °C in 5% CO<sub>2</sub>. The transfection medium was then replaced with normal growth medium and 36 h later supplemented with the respective drugs for selection of stable cell lines. SK-OV-3 cells stably expressing tetracycline-regulated HER-2/neu-targeted ribozymes were generated in a two-step transfection protocol. In the first step, SK-OV-3 cells were transfected with 10 µg of pUHG15-1 plasmid DNA and 1 µg of pcDNA3 plasmid DNA (Invitrogen, San Diego, CA) to provide G418 resistance. After selection for stable cell lines in the presence of G418 at 0.7 mg/ml, individual tTA-expressing clones were isolated with cloning rings. To test the clones for tTA expression and tetracycline regulation, the cells were transiently transfected in the absence and presence of 1 µg/ml tetracycline (Sigma) with pUHC13-3 plasmid DNA containing a luciferase cDNA under the control of the tet-O-binding site (15). Cell lysates were prepared 36 h after the transfections, and luciferase activities were measured in a luminometer as described (17). Two clones (SK-OV-3/tTA-7 and SK-OV-3/tTA-2) that demonstrated the best tetracycline regulation of luciferase activity were used for further transfections with the ribozyme expression plasmids. SK-OV-3/tTA-7 and SK-OV-3/tTA-2 cells were then transfected with 10 µg of pTET/Rz3, pTET/Rz3mu, or pTET/Rz4 mixed with 1 µg of pZero (Invitrogen) to provide Zeocin resistance. Mass-transfected derivative cell lines (SK-OV-3/Rz3, SK-OV-3/Rz3mu, and SK-OV-3/Rz4) were obtained after selection with 0.4 mg/ml Zeocin and 1 µg/ml tetracycline.

**Northern Analysis**—Total cellular RNA was isolated with the RNA STAT-60 method (Tel-Test, Friendswood, TX), and 30 µg were separated and blotted as described (19). A HER-2/neu cDNA probe (1.5-kilobase pair *Eco*RI fragment) was hybridized, washed, and exposed to film for 16 h (19). To correct for variability in loading, blots were stripped, reprobed with a glyceraldehyde-3-phosphate dehydrogenase cDNA probe (CLONTECH), and exposed to film for 1 h. Relative band intensities were measured by densitometry.

**Fluorescence-activated Cell Sorting (FACS)**—To quantitate HER-2/neu protein levels by FACS analysis, cells were trypsinized, washed once with growth medium containing serum and twice with phosphate-buffered saline (Sigma), and resuspended in phosphate-buffered saline at  $5 \times 10^5$  cells/100 µl. The cells were incubated for 30 min at 4 °C with a 1:100 dilution of a primary anti-human HER-2/neu mouse monoclonal antibody (clone 9G6.10; Neomarkers, Fremont, CA). Cells were washed twice with phosphate-buffered saline and incubated for 30 min at 4 °C in the dark with a 1:200 dilution of a fluorescein isothiocyanate-labeled goat anti-mouse secondary antibody (Boehringer Mannheim). After two final washes with phosphate-buffered saline, the mean value of fluorescence intensity of 10,000 cells was determined by FACS (FACStar Plus, Becton Dickinson). Unlabeled cells and cells labeled with secondary antibody alone served as negative controls.

**Tumor Growth in Animals**—Female athymic nude mice (NCr nu/nu; NCI, Frederick, MD) were injected subcutaneously with  $1 \times 10^6$  cells in 100 µl of Iscove's modified Eagle's medium (three mice/group and two

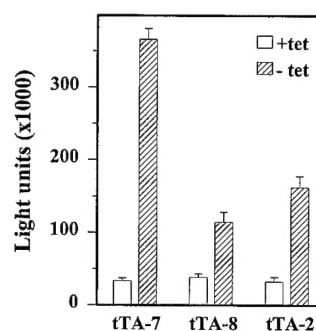


Fig. 1. Three stably transfected SK-OV-3 clones (SK-OV-3/tTA-7, SK-OV-3/tTA-8, and SK-OV-3/tTA-2) that express the tTA protein were transiently transfected with pUHC13-3 plasmid DNA, coding for luciferase under the control of the tet-O-binding site (13). Luciferase activity was measured 36 h after transfection in the absence and presence of tetracycline (tet).

injection sites/mouse). Mice in group 1 were injected with SK-OV-3/tTA-7 control cells, and those in groups 2 and 3 with SK-OV-3/Rz3 ribozyme-expressing cells. In the mice in group 3, slow-release tetracycline pellets (Innovative Research of America), which release 0.7 mg of tetracycline/day, were implanted subcutaneously at the day of tumor cell injection. Tumor growth was monitored for up to 2 months, and tumor sizes were estimated from the product of the perpendicular diameters of the tumors. In one mouse in group 3, the tetracycline pellet was removed after 6 weeks to activate ribozyme expression in established tumors, and tumor growth was monitored for an additional 2 weeks. In a separate study, nude mice were injected subcutaneously with  $2 \times 10^6$  SK-OV-3/Rz4 cells in 100 µl of Iscove's modified Eagle's medium (five mice/group and two injection sites/mouse). Tumor growth was monitored for 4 weeks in the presence (group 1) or absence (group 2) of tetracycline.

**Data Analysis**—Means  $\pm$  S.E. are depicted unless indicated otherwise. Student's *t* test or analysis of variance for repeated measures (Statview 4.02, Abacus Concepts, Inc.) was used for comparisons between data sets, and  $p < 0.05$  was considered significant.

## RESULTS

**Generation of tTA-expressing SK-OV-3 Cells**—To avoid promoter interference and to generate cells in which tet-O-controlled transgene expression can be tightly regulated by tetracycline, a two-step transfection protocol was used as originally described (15). In the first step, SK-OV-3 cells were transfected with pUHG15-1 plasmid DNA, and individual clones were screened for tetracycline regulation of tTA-driven tet-O/luciferase expression. Individual tTA-expressing clones were transiently transfected with pUHC13-3 plasmid DNA, coding for luciferase under the control of the tet-O-binding site, and luciferase activity was measured in the absence and presence of tetracycline. In the absence of tetracycline, luciferase activity was high in all clones. The best tetracycline regulation was observed in clone 7 (SK-OV-3/tTA-7), where tTA-driven luciferase activity was repressed by >90% in the presence of tetracycline (Fig. 1). Clones SK-OV-3/tTA-7 and SK-OV-3/tTA-2 were then used for further transfections with pTET/ribozyme expression plasmids. It is important to note that luciferase activity was not completely repressed to background levels, thus demonstrating some residual promoter activity in these cells. HER-2/neu expression as measured by FACS analysis did not vary between the clones, indicating that clonal selection and/or tTA expression had no significant effects on HER-2/neu expression in SK-OV-3 cells (data not shown).

**Efficacy of HER-2/neu-targeted Ribozymes in Cells in Vitro**—SK-OV-3/tTA-7 cells were transfected with pTET/Rz3 or pTET/Rz3mu plasmids, and mass-transfected cell pools were selected in the presence of tetracycline to repress ribozyme expression during the selection period. After 6 weeks, stably transfected cells were grown in the absence or presence of tetracycline, and HER-2/neu mRNA levels were quantified by

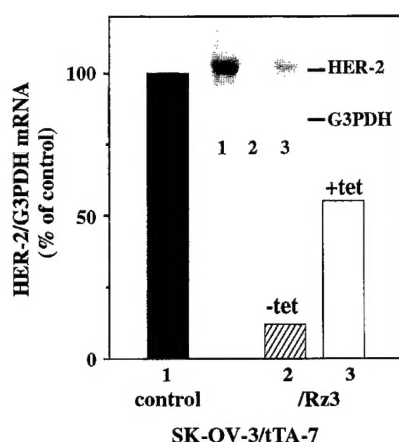


FIG. 2. Total RNA (30  $\mu$ g) from SK-OV-3/tTA-7 control cells (lane 1) and anti-HER-2/neu-targeted R3 ribozyme-transfected cells grown in the absence (lane 2) and presence (lane 3) of tetracycline (tet) was analyzed by Northern analysis. HER-2/neu mRNA was quantified relative to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA by densitometry. The inset shows a representative Northern blot. The positions of HER-2/neu- and glyceraldehyde-3-phosphate dehydrogenase-specific transcripts are indicated. The HER-2/neu Northern blot was exposed to film for 16 h, whereas the glyceraldehyde-3-phosphate dehydrogenase Northern blot was exposed for only 1 h.

Northern analysis (Fig. 2). HER-2/neu-targeted pTET/R3 ribozyme expression (Fig. 2, lane 2 (-tet = ribozyme on)) depleted endogenous HER-2/neu mRNA levels by 90% compared with control cells. Inactivation of ribozyme expression (Fig. 2, lane 3) was followed by a 4.5-fold increase in HER-2/neu mRNA levels, demonstrating the specificity of the ribozyme effect. Expression of the pTET/R4 ribozyme construct was less efficacious and reduced HER-2/neu mRNA levels in a tetracycline-reversible manner by ~50% (data not shown).

In the next experiment, ribozyme-mediated depletion of HER-2/neu protein expression was assessed by FACS analysis. Cell-surface expression of the HER-2/neu protein was suppressed by 90% when cells were grown in the absence of tetracycline (Fig. 3A, R3-on). HER-2/neu levels almost completely reverted to control values when tetracycline was added to the medium. In addition, a catalytically inactive mutant ribozyme (SK-OV-3/R33mu) had no significant effects on HER-2/neu mRNA or protein expression (data not shown), which clearly indicates that cleavage of HER-2/neu RNA and not antisense inhibition is the main mode of ribozyme action. Expression of pTET/R4 reduced HER-2/neu protein levels by 50% (Fig. 3B), correlating very well with the results from the Northern analysis.

In parallel experiments with other cell lines, we verified the efficacy of the anti-HER-2/neu-targeted ribozymes. Ribozyme targeting caused a reduction of HER-2/neu expression in MKN7 gastric cancer, MDA-MB-361 breast cancer, and Colo357 pancreatic cancer cells (data not shown). This strongly suggests that this targeting approach will be useful in a variety of human adenocarcinomas.

The biological significance of a HER-2/neu depletion on *in vitro* growth of SK-OV-3 cells was assessed by anchorage-dependent as well as anchorage-independent growth assays. HER-2/neu-targeted ribozyme expression did not alter cell morphology or anchorage-dependent proliferation. However, anchorage-independent growth in soft agar was inhibited by >90% in SK-OV-3/R3 cells and was restored to control levels by the addition of tetracycline (data not shown). This demonstrates that HER-2/neu is a rate-limiting factor for anchorage-independent growth of SK-OV-3 cells.

*Down-regulation of HER-2/neu Expression Inhibits Tumor*

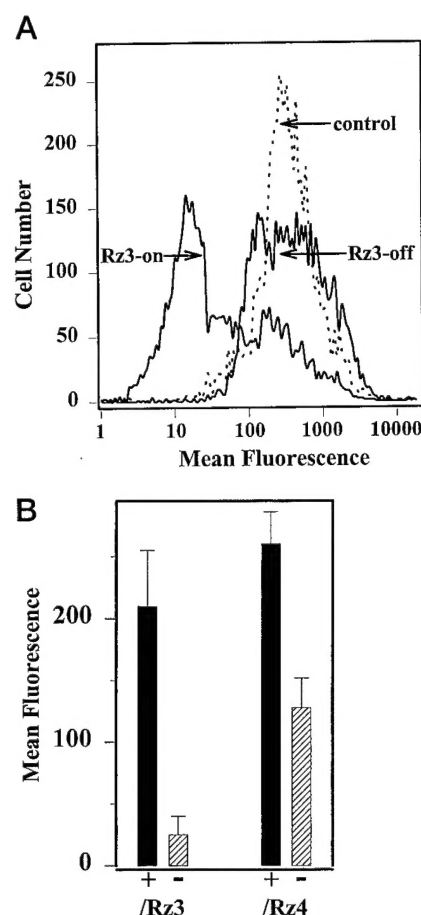
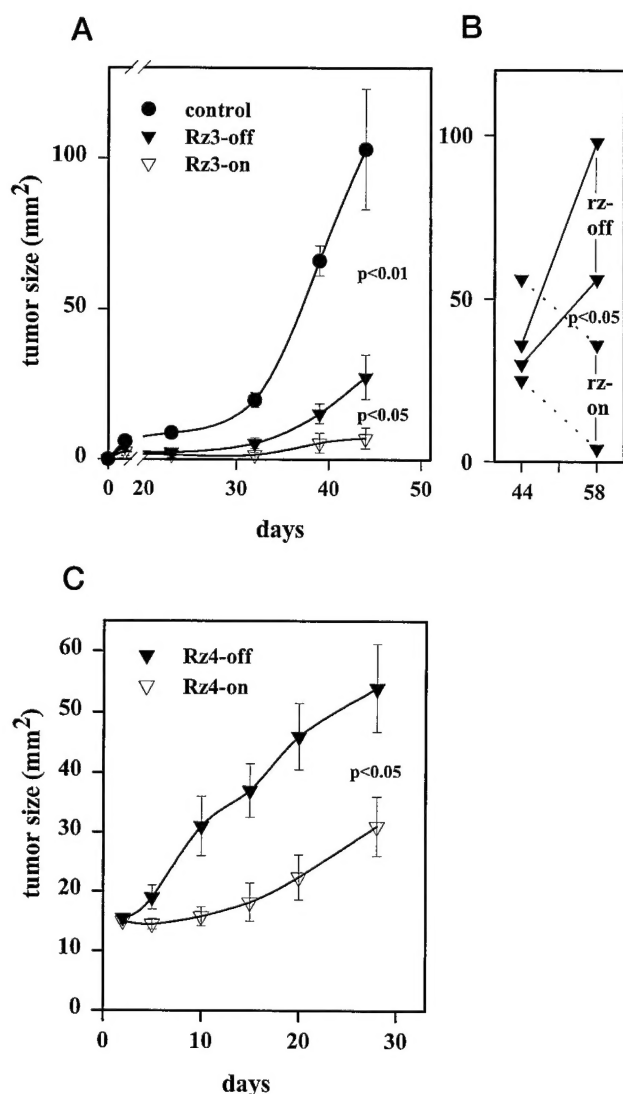


FIG. 3. A, FACS analysis of HER-2/neu protein expression in SK-OV-3/tTA-7 control cells (dotted line; mean  $\pm$  S.D. =  $240 \pm 25$ ) compared with SK-OV-3/R3 ribozyme-expressing cells (solid lines) in the absence (R3-on) and presence (R3-off) of tetracycline. B, mean fluorescence values of FACS analysis of HER-2/neu protein expression in SK-OV-3 cells transfected with two different ribozyme constructs (pTET/R3 and pTET/R4) in the absence (-; ribozyme on) and presence (+; ribozyme off) of tetracycline.

**Growth in Vivo in Nude Mice**—When injected subcutaneously at  $1 \times 10^6$  cells/injection site into nude mice, SK-OV-3/tTA-7 control cells grew to a mean tumor size of  $100 \pm 20$  mm<sup>2</sup> within 44 days (Fig. 4A, ●). In contrast, tumor growth of ribozyme-expressing pTET/R3 cells was significantly inhibited ( $p < 0.01$ , Student's *t* test), and tumors did not grow beyond a very small inoculum size of  $6 \pm 2.6$  mm<sup>2</sup> (Fig. 4A, ▽). In animals that were treated with slow-release tetracycline pellets to turn the ribozyme off *in vivo*, tumor growth started to increase after 32 days, and mean tumor sizes were  $27 \pm 7.8$  mm<sup>2</sup> (Fig. 4A, ▲; significantly different from ribozyme-expressing tumors,  $p < 0.05$ , Student's *t* test). Tumor growth of SK-OV-3 cells transfected with the catalytically inactive ribozyme (pTET/R3mu) was not significantly different compared with control cells (data not shown).

In a subset of this study, we addressed the question of whether ribozyme-mediated abrogation of HER-2/neu expression can cause regression of established tumors. Ribozyme expression was activated in the tumor cells by removal of the tetracycline pellet after 44 days, and tumor growth was monitored for another 2 weeks. While the tumors in the tetracycline-treated animals (ribozyme off) grew continuously and reached a size of  $76 \pm 20$  mm<sup>2</sup> (Fig. 4B, solid lines), the tumors in which the ribozyme was activated by removal of tetracycline started to regress and reached a size of  $20 \pm 3.5$  mm<sup>2</sup> (Fig. 4B, dotted lines). This tumor regression was significant ( $p < 0.05$ , analysis





**FIG. 4. Subcutaneous tumor growth in nude mice of SK-OV-3 ovarian cancer cells producing different levels of HER-2/neu.** A,  $1 \times 10^6$  cells were inoculated subcutaneously (three mice/group and two injection sites/mouse), and tumor growth was monitored for 6 weeks in SK-OV-3/TA control cells (●) or pTET/Rz3 ribozyme-transfected cells in the presence (Rz3-off; ▼) and absence (Rz3-on; ▽) of tetracycline treatment. B, after 44 days, growth curves of the individual tumors in the absence (rz-on; dotted lines) and continued presence (rz-off; solid lines) of tetracycline were monitored for another 2 weeks. C, SK-OV-3 cells transfected with the pTET/Rz4 ribozyme expression construct were inoculated subcutaneously at  $2 \times 10^6$  cells/injection site (five mice/group and two injection sites/mouse), and tumor growth was monitored for 4 weeks in the presence (Rz4-off; ▼) and absence (Rz4-on; ▽) of tetracycline.

of variance for repeated measures) and suggests that HER-2/neu expression is important for continuous growth of ovarian cancer cells *in vivo*. Furthermore, disruption of HER-2/neu not only arrested tumor growth, but induced regression.

To test if SK-OV-3 tumor growth correlates with the level of HER-2/neu expression, we used SK-OV-3/Rz4 cells, in which, in contrast to SK-OV-3/Rz3 cells, HER-2/neu protein levels are reduced by ~50% (see Fig. 3B). When injected subcutaneously at  $2 \times 10^6$  cells/injection site in the presence of tetracycline, SK-OV-3/Rz4 cells grew to a mean tumor size of  $54 \pm 7.2$  mm<sup>2</sup> within 4 weeks (Fig. 4C, ▼). Inactivation of ribozyme expression by tetracycline significantly inhibited tumor growth by 43% (Fig. 4C, ▽;  $p < 0.05$ , Student's *t* test), which correlated very well with the level of HER-2/neu reduction in these cells.

The overall faster tumor growth in this experiment compared with the previous study (see Fig. 4A) was due to the higher tumor cell inoculum ( $2 \times 10^6$  versus  $1 \times 10^6$ ). This result further supports the thesis that HER-2/neu expression is rate-limiting for SK-OV-3 tumor growth in a gene dose-dependent manner.

#### DISCUSSION

The phenomenology of HER-2/neu overexpression in human cancer has been well studied, and particularly the easily accessible cell-surface localization made it an excellent target for antibody-based immunotherapies. HER-2/neu-specific monoclonal antibodies (20) and recombinant immunotoxins (21), both of which can inhibit *in vitro* and *in vivo* growth of transformed cells, have been described. Despite the clinical usefulness of these approaches, it remains unclear how expression of an apparently normal gene product affects tumorigenesis and tumor progression *in vivo*. Potentially more specific genetic targeting strategies such as antisense oligonucleotides have been used with limited success (22), probably due to their relatively low efficacy and specificity.

In this study, we expressed HER-2/neu-targeted hammerhead ribozymes under the control of a tetracycline-regulated promoter system in human SK-OV-3 ovarian cancer cells, which express HER-2/neu spontaneously at high levels due to gene amplification. SK-OV-3 cells provide an attractive model since the epidermal growth factor receptor is the only other human epidermal growth factor receptor expressed in these cells, which therefore proliferate in response to epidermal growth factor, but not to new differentiation factor/hereregulin-like growth factors (data not shown).

To achieve a conditional HER-2/neu down-regulation, we employed a binary tetracycline-regulated gene expression system in which hammerhead ribozyme expression can be inactivated *in vitro* and *in vivo* by the addition of the nontoxic antibiotic tetracycline. The major advantage over a constitutive ribozyme expression system is that the effects of ribozyme-mediated down-regulation of HER-2/neu expression can be evaluated in one genetically identical cell line, thus preventing interferences of different genetic backgrounds between various cell lines. Furthermore, HER-2/neu can be down-regulated at different time points in tumor growth in animals, which allows the identification of the tumor stages that are most susceptible to anti-HER-2/neu-targeted therapies.

In stably mass-transfected SK-OV-3 cells, Rz3 ribozyme expression depleted HER-2/neu mRNA and protein levels by >90%, and inactivation of ribozyme expression by tetracycline reversed the effects. This inhibition is even more remarkable taking into account that mass-transfected cells (and not clonal subpopulations) were used and that HER-2/neu is expressed at very high levels in SK-OV-3 cells. This suggests that ribozymes can be more effective than antisense oligonucleotides that reduced HER-2/neu expression by only 50% in selected clones of SK-BR-3 breast cancer cells (22), which express HER-2/neu at comparable levels. This dramatic inhibition enabled us for the first time to study the effects of a functional and conditional HER-2/neu knockout on *in vivo* tumor growth of ovarian cancer cells. Tumor growth of ribozyme-expressing cells was almost completely abrogated, and inactivation of ribozyme expression by tetracycline partially reversed SK-OV-3 tumor growth. Inactivation of ribozyme expression by tetracycline was not as effective as in the cell culture experiments, which can potentially be explained by an insufficient tetracycline delivery to the tumor cells *in vivo*. Alternatively, *in vivo* tumor growth might be more susceptible to residual ribozyme activity and hence small reductions in HER-2/neu expression.

In summary, our data demonstrate that HER-2/neu has a

rate-limiting role in ovarian cancer and that ribozyme targeting can cause regression of established tumors. The conditional *HER-2/neu* depletion *in vitro* and *in vivo* will enable further elucidation of the role of *HER-2/neu* in the pathogenesis of human cancer.

## REFERENCES

1. Bacus, S. S., Zelnick, C. R., Plowman, G., and Yarden, Y. (1994) *Am. J. Clin. Pathol.* **102**, S13-S24
2. Bargmann, C. I., Hung, M. C., and Weinberg, R. A. (1986) *Cell* **45**, 649-657
3. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W., Ullrich, A., and McGuire, R. L. (1987) *Science* **235**, 177-182
4. Di Fiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R., and Aaronson, S. A. (1987) *Science* **237**, 178-182
5. Stancovski, I., Sela, M., and Yarden, Y. (1994) in *Regulatory Mechanisms of Breast Cancer* (Lippman, M. E., and Dickson, R. B., eds) pp. 161-191, Kluwer Academic Publishers, Boston, MA
6. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., and Ullrich, A. (1989) *Science* **244**, 707-712
7. Karunagaran, D., Tzahar, E., Beerli, R. R., Chen, X., Graus-Porta, D., Ratzkin, B. J., Seger, R., Hynes, N. E., and Yarden, Y. (1996) *EMBO J.* **15**, 254-264
8. Beerli, R. R., Wels, W., and Hynes, N. E. (1994) *J. Biol. Chem.* **269**, 23931-23936
9. Graus-Porta, D., Beerli, R. R., and Hynes, N. E. (1995) *Mol. Cell. Biol.* **15**, 1182-1191
10. Christoffersen, R. E., and Marr, J. J. (1995) *J. Med. Chem.* **38**, 2023-2037
11. Czubayko, F., Schulte, A. M., Berchem, G. J., and Wellstein, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 14753-14758
12. Feng, M., Cabrera, G., Deshane, J., Scanlon, K. J., and Curiel, D. T. (1995) *Cancer Res.* **55**, 2024-2028
13. Scanlon, K. J., Jiao, L., Funato, T., Wang, W., Tone, T., Rossi, J. J., and Kashani-Sabet, M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 10591-10595
14. Jarvis, T., Alby, L., Beaudry, A., Wincott, F., Beigelman, L., McSwiggen, J., Usman, N., and Stinchcomb, D. (1996) *RNA* **2**, 419-428
15. Gossen, M., and Bujard, H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 5547-5551
16. Jones, J., Lagasse, L. D., and Karlan, B. Y. (1994) *Gynecol. Oncol.* **55**, 421-426
17. Schulte, A. M., Lai, S., Kurtz, A., Czubayko, F., Riegel, A. T., and Wellstein, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 14759-14764
18. Czubayko, F., Riegel, A. T., and Wellstein, A. (1994) *J. Biol. Chem.* **269**, 21358-21363
19. Fang, W. J., Hartmann, N., Chow, D., Riegel, A. T., and Wellstein, A. (1992) *J. Biol. Chem.* **267**, 25889-25897
20. Hurwitz, E., Stancovski, I., Sela, M., and Yarden, Y. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 3353-3357
21. Chen, S. Y., Yang, A. G., Chen, J. D., Kute, T., King, C. R., Collier, J., Cong, Y., Yao, C., and Huang, X. F. (1997) *Nature* **385**, 78-80
22. Vaughn, J. P., Iglehart, J. D., Demirdji, S., Davis, P., Babiss, L. E., Caruthers, M. H., and Mark, J. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 8338-8342